Oxidative stress and apoptotic biomarkers in human semen

Reda Zakria Mahfouz

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Oxidative stress and apoptotic biomarkers in human semen

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Doctoral thesis

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by

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Chapter 1

Introduction

A clinical-pathology review of apoptosis and oxidative stress in reproduction

Introduction

Assisted reproductive techniques (ART) have offered the possibility of treatment in many infertile couples with male factor infertility. However, success is not guaranteed and success rates for ART procedures still remain suboptimal [1]. Originally developed to combat female infertility, ART practice is increasingly important in andrology. The use of spermatozoa from ejaculates with poor spermiogram or from non-physiological sources such as the epididymis and testis raises a number of concerns. It is for instance possible that some sperm selected for ART will display features of damage at the molecular levels despite they appear normal, which may be partially responsible for low blastocyst development rates (BDR), pregnancy rates (PR) and recurrent pregnancy loss (RPL) [2, 3]. The negative effects of sperm damage, specifically DNA fragmentation appear during genome activation after fertilization [4].

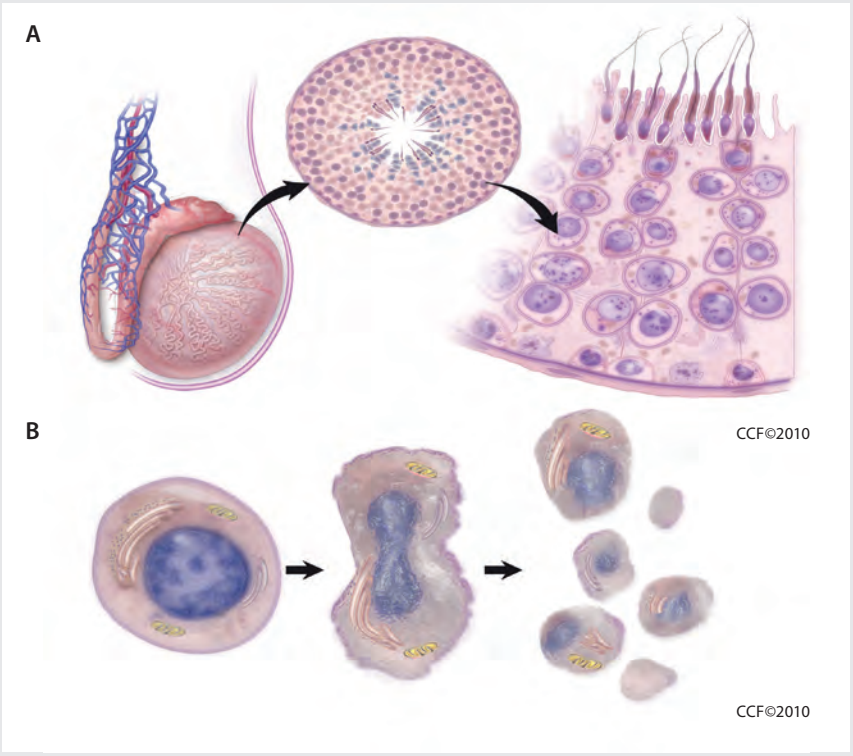
Programmed cell death (apoptosis) is a cascade of cellular events that are under genetic control and leads to a series of cellular, morphological and biochemical changes away from healthy homeostasis culminating into cellular suicide [5] (**Figure** 1a-c). Reports link the presence of apoptotic markers in human sperm with the failure of in-vivo as well as in-vitro fertilization [3, 6, 7]. Externalization of phosphatidylserine (PS) from the inner leaflet (its normal location) in the sperm plasma membrane to its outer surface is one of apoptotic reversible changes observed in human spermatozoa which becomes irreversible when it is accompanied with extensive DNA damage [8, 9]. Origin of sperm DNA damage can be explained mainly by defective spermatogenesis and OS mechanisms [10]. Sperm DNA fragmentation is a critical apoptotic feature that has been identified in ejaculated spermatozoa [11, 12]. Reports showed the link between OS-induced sperm damaged and the presence of apoptotic changes especially as to DNA damage [13, 14]. Developing new protocols for sperm diagnostic assays based on the early detection of OS markers and/ or apoptosis-like changes should be considered as tools to improve ART success rates and to optimize reproduction lab services.

In order to better comprehend the different aspects of OS impact in spermatozoa damage and clinical results described in this thesis, a general description of spermatogenesis, OS and apoptotic markers in mature sperm, the tools used to measure them and potential laboratory applications, are addressed in this chapter.

1. Spermatogenesis overview

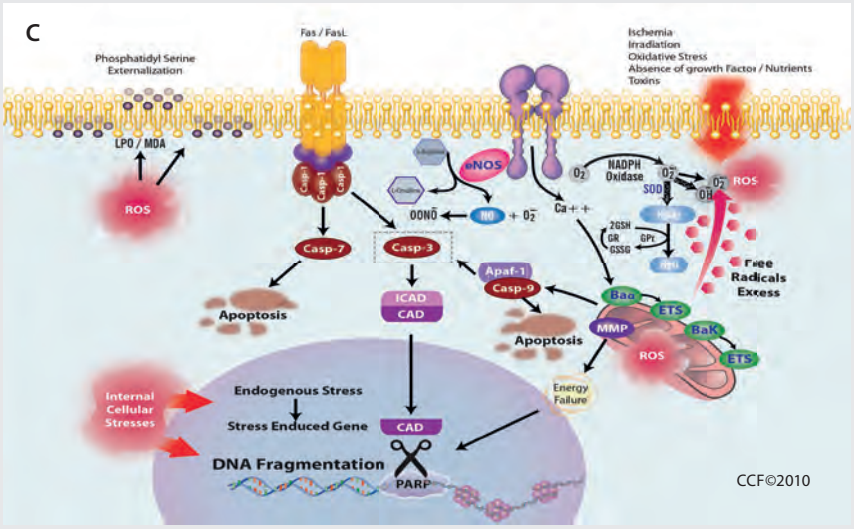
Spermatogenesis is the complex process of highly organized self renewal/ proliferation and cyclic maturation leading to germ cells differentiation. It includes specific morphological cellular associations of the seminiferous epithelium, and actions of FSH/ testosterone to control the continuous differentiation of the germ cells to mature haploid motile

Figure 1 **(A)** Gross structure of testis shows distribution of the seminiferous tubules collecting together to end in the epididymis, then vas deferens. Spermatic vessels plexus forms the main components of spermatic cord. Tortuous spermatic venous plexus observed in infertile men and is considered the most common pathologies in male infertility. Magnified cross section in a seminiferous tubule is to show the diameter and its lining of the anatomical unit of spermatogenesis. Seminiferous tubules as further studied longitudinally to show its lining layer of Sertoli cells with differentiating germ cells maturation stages **(B)** Morphological changes of germ cells which occur in response to injurious agents such as apoptosis.



spermatozoa [15, 16]. Impaired spermatogenesis accounts for male factor infertility which approximately forms 50% of all causes in infertile couples [17, 18]. This impairment translates into defective sperm function. Molecular changes, may start even in the early maturation stages the spermiogenesis, which result in production of functionally unhealthy spermatozoa[19]. Concomitantly, there can be partially arrested spermatogenesis, depending on severity, type, or level of the affected molecular pathways. Environmental factors such as infections [20] life style stresses [21, 22] and varicocele [23, 24] may interfere with normal and impaired spermatogenesis by causing or aggravating OS. This type of damage may occur in early and late stages of spermatogenesis [25, 26].

Figure 1 **(C)** Activation of the most common pathways for oxidative stress (OS) and apoptosis in somatic cells which may get activated in response to external or internal cellular injurious stimuli. There may be activation of interacting pathways which favor OS induced damage with apoptotic machinery activation. These interactions cause energy failure and augment DNA fragmentations and suppression of DNA repair. (ETS, electron transfer system; MMP, mitochondrial membrane potential; GR, glutathione reductase; GPx, glutathione perioxidase; SOD, superoxide dismutase; Bax/Bak, proapoptotic proteins essentials for mitochondrial induced apoptosis; eNOS, endothelial nitric oxide synthase; CAD, caspase activated DNase; ApoF1, apoptosis inducing factor; PARP, poly (ADP)-ribose polymerase; Cas, caspase)



2. Sperm integrity in male reproduction

Sperm chromatin integrity is an important factor as male gametes are main contributors in embryonic development [4, 27]. In normal spermatogenesis, sperm chromatin is a highly organized, compact structure consisting of DNA and nucleoproteins [28]. Human sperm chromatin is different from other animal species as they have a higher proportion of histones and percents of high heterogeneity within the same specimen. Sperm chromatin is condensed in an organized way to protect and facilitate a safe transport of the paternal genome to the ready mature oocyte through fertilization process [29]. Integrity of sperm chromatin is a result of multiple molecular and morphological maturation steps completed with production of healthy sperm. Reports showed that protamination status, zinc, vitamin A, disulphide bonds and DNA breaks may collectively or individually have a role in sperm chromatin quality [30-32]. Sperm chromatin integrity has been extensively studied recently to explore its significance in clinical reproduction laboratory [33] .

The presence of (faulty) DNA repair in early embryonic development is related to the origin of gene mutation. The human cellular genome is almost by default protected by mechanisms collectively known as DNA repair activity. DNA damage is critical in early human life, especially during fertilization as well as in early embryonic development.DNA repair activity in the zygote is mandatory in order to avoid mutation induction in the germ line [27].The current viewpoint is that DNA repair is a maternal trait. DNA repair activity may be affected by the transcripts stored during maturation processes of the oocyte [34, 35].

3. Oxidative stress (OS) and apoptosis in human spermatozoa

The main sources of DNA damage in sperm are either apoptosis or reactive oxygen species (ROS) [36, 37]. Sperm DNA damage is indicated to be of concern in male infertility and/ or ART [33, 38], because of the genetic anomalies that can be induced in embryos [39, 40].

3.1 Oxidative Stress (OS)

OS reflects a status which results from the imbalance of generating ROS beyond the available antioxidant capacity within gametes, their progenitors or embryonic cells [13, 41]. In sperm, there is an inability to readily detoxify the reactive intermediates and/ or to repair the resulting OS induced damage within limited capabilities. The affected cells may show markers for damage of their protein, lipids, or DNA molecular compounds [13, 42].

The presence of non-physiological high levels of ROS generation in male reproductive tract are potentially toxic to sperm quality and function [43]. Human spermatozoa are particularly susceptible to OS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids (PUFA) while their scanty cytoplasm contains low concentrations of scavenging enzymes [44]. High levels of seminal ROS are reported in 25% to 40% of infertile men [45]. Although the source(s) by which human spermatozoa generate intra-cellular ROS remain to be clarified, there may be a significant role for the enzyme β-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in generating ROS [46].

Two factors protect the sperm DNA from oxidative insult: the characteristic packaging of the DNA by protamines and the available TAC in seminal plasma [47]. However, OS may develop as a result of an imbalance between ROS generation and scavenging activities [48]. Strong evidence suggests that high levels of ROS mediate the occurrence of high frequencies of single- and double-strand DNA breaks commonly observed in the spermatozoa of infertile men [49]. Studies in which the sperm was exposed to artificially high ROS conditions showed a significant increase in DNA damage Furthermore, ROS play an important role in mediating apoptosis by inducing cytochrome *c*

and caspases 9 and 3 activation, which in turn result in a high frequency of single and double stranded DNA strand breaks [50].

3.1.1 Markers of OS Induced Damage

OS markers include seminal ROS, seminal plasma TAC, and sperm damage markers. These markers reflect oxidative damage to the lipid, protein or nucleotide components of spermatozoa which impair their function [51]. Teratozoospermia occurs as a result of defective spermatogenesis and is characterized by an abundance of spermatozoa carrying surplus residual cytoplasm. The retention of residual cytoplasm promotes spermatozoa to generate endogenous ROS via mechanisms that may be mediated by the cytosolic G6PD (Glucose 6-phosphate dehydrogenase) enzyme [52]. Therefore, patients presenting with teratozoospermia are at greater risk of developing pathogenic levels of ROS, and subsequently sperm DNA damage.

In general, ROS production is highest in immature spermatozoa from males with abnormal semen parameters and appears to be associated with high levels of DNA damage [53]. However, spermatozoa with cytoplasmic retention are not the only abnormal cells that are associated with high levels of DNA damage and high ROS production. Spermatozoa with abnormal head morphology, midpiece defects and tail defects also display the same characteristics. Production of ROS positively correlates with sperm deformity index (SDI), which is calculated by dividing the total number of deformities observed by the number of sperm evaluated [54]. Similarly, spermatozoa with large nuclear vacuoles were shown to present with higher levels of DNA fragmentation [55].

3.2 Apoptotic pathways during spermatogenesis

Mature human spermatozoa result from unique differentiation/ maturation processes of germ cell progenitors. Germ cell progenitors are supported and nourished by Sertoli cells. Testicular blood barrier protects early germ cell progenitors from being attacked from external injurious agents or their own immune system [56]. Disruption of this barrier by trauma, surgery or infection may induce germ cell stresses [57]. Depending upon the repair capacity and severity of injurious factors will be de degree of the affection on spermatogenesis. To directly or indirectly evaluate apoptotic effects on sperm quality, a number of molecular markers were proposed to detect in (aberrant) spermatozoa [10]. They include structural changes in surface (e.g. phosphatidyl serine (PS) externalization), cytoplamsic proteins (e.g. caspases) or nuclear markers (e.g. DNA breaks) which could be detected through specialized assays that may help in detecting spermatogenesis abnormalities in some infertile men [58]. **Figure 1a** shows testis, anatomical sections with germinal epithelium differentiation in normal conditions. The most common apoptotic pathways which may be activated in germinal epithelium (and the involved molecules) in response to injuries are demonstrated in Figure 1 C.

Apoptotic pathways in mature spermatozoa remain not fully understood because of almost absence of cytoplasm and reduced nuclear functional activities. The most accepted theory is "abortive apoptosis by Sakkas"[59]. Other possible pathway for DNA damage is activation of endonucleases by external DNA [60].

3.3 Apoptotic Markers in Sperm

The use of non-apoptotic spermatozoa for reproduction is imperative for achieving successful fertilization that should be followed by subsequent good embryo quality. As compared to somatic and testicular germ lines, the significance of apoptosis phenotype in ejaculated spermatozoa remains controversial [61]. Apoptosis-related features, reported in human spermatozoa, may indicate anomalies in the regulation of apoptosis in the testis [11]. Abortive apoptosis is a theory which proposes failure to eliminate abnormal spermatozoa during spermatogenesis differentiation stages which may explain their presence in ejaculated semen [59, 62]. The use of these damaged spermatozoa may compromise the fertilization potential (however, damaged sperm may fertilize oocyte), affecting the early embryo development [4, 27]. Depending upon the degree of damage, early apoptosis can be reversible when the plasma membrane is only affected and shows a repairable damage. Late apoptosis is considered when damage becomes un-repairable following extensive or nuclear damage [63].

The cellular changes that may be present in sperm, which are induced by apoptosis or OS induced damage, are associated with molecular markers which can be used for diagnostic or therapeutic applications. In the next paragraph, the most used molecular markers are described.

A. Early apoptotic markers

i. Externalization of phosphatidylserine (PS)

PS is a phospholipid located on the inner leaflet of the plasma membrane. PS has a high and selective affinity for annexin V, a 35-36 kD phospholipid binding protein Annexin-PS binding occurs after translocation of PS from inner to outer leaflets of plasma membrane resulting in externalization of PS (EPS) on the surface. Translocated PS is one of the earliest detectable features of cells undergoing apoptosis [64]. EPS negatively correlates with the sperm quality. Reduced integrity of sperm membrane is more frequently seen in spermatozoa from infertile men that contribute to childlessness [65].

ii. Mitochondrial dysfunction

Sperm mitochondria are susceptible to injurious agents' apoptotic stimuli due to their compartmentalization within the midpiece region. Intact mitochondrial membrane potential (MMP) is determined to be essential for sperm motility [66]. MMP disruption is considered as a key marker for apoptosis signaling cascade which is observed in human spermatozoa. A strong correlation could be found between MMP and DNA

fragmentation levels in human spermatozoa [67]. In support of the implication of apoptosis in human reproduction, EPS, mitochondrial dysfunction, and nuclear DNA damage were detected in significantly higher levels in infertile men and those with varicocele [68].

B. Late Apoptotic Markers

i. Caspase (s) activation

Caspase (CP) activation is believed to be a well-defined point of no return for apoptosis progression, and a number of apoptotic events downstream of caspase activation have been characterized among which DNA fragmentation stands as a critical apoptotic event [69]. Evidence supports that within the testicular cells, caspases play a central role in the apoptotic process that leads to DNA fragmentation of Sertoli cells [70]. Activated CP-3 induces activation of caspase-activated deoxyribonuclease (CAD; also called caspase-activated nuclease), which is integrally involved in degrading DNA. Therefore, CP-3 executes the final disassembly of the cell by generating DNA strand breaks [71] (**Figure** 1c).

Samples from infertile patients are characterized by high numbers of cells with activated caspases, especially in those with cytoplasmic droplets, with a strong positive correlation to EPS. Presence of precursors and activated forms of CP-8 and CP-9 in conjunction with CP-3 in human spermatozoa has also been confirmed [72].

ii. DNA fragmentation

Sperm DNA fragmentation was found to be prevalent in fractions of sperm with positive immunostaining for CP-3, suggesting a relation between them [12]. Further, a significant positive correlation was seen between activated CP-3 levels in the sperm midpiece and DNA fragmentation in specimens with low motility, suggesting that caspase-dependent apoptotic mechanisms could originate in the cytoplasmic droplet or within mitochondria and function in the nucleus [73] (**Figure 1)**. In addition, DNA damage seems to be correlated with abnormal sperm morphology and low motility [74]. DNA damage can be directly measured by TUNEL or Comet assays which have been discussed below (**Figure** 2).

4. Laboratory role in human reproduction

Management of infertile couples requires an accurate diagnosis for optimizing the chances to pregnancy and overcome repeated treatment failures [75]. Infertility/ sub-fertility is a common health problem that can be addressed successfully with a number of interventions. The physician must be careful when concluding male infertility diagnosis based only on the routine sperm parameters as concentration, motility, and especially morphology [76]. Idiopathic infertility, where no abnormalities in

neither male nor female investigations are found, might in part caused by molecular OS-induced damages. Improvement of the quality of andrology lab preparation techniques such as sperm selection and cryobiology procedures for future use of cryopreserved gametes or embryos is imperative. Iatrogenic damage to spermatozoa by sperm preparation techniques should be avoided when possible [47].

4.1 Routine Semen Analysis

Semen is a reflection of the functional capacity of different components of the male reproductive system. The standard semen analysis will remain the corner stone for male infertility diagnosis. Standard semen analysis includes measurement of semen volume, pH, sperm concentration, motility percentage (and total motile, and progressive motile), leukocytes and sperm antibody assessment [76, 77]. Two main systems are recommended for sperm morphology assessment, WHO and Strict's criteria. Although sperm morphology has little prediction on the ART outcome, pregnancy rate/cycle with ART reported to decline from 30% in patients with normal sperm morphology to 20 % with the use of compromised sperm morphology according to WHO guidelines [78] (**Figure** 2-A show routine sperm examination in healthy and infertile men).

4.2 Additional Assays for Semen Quality

There are several assays that may have the merit to be used for detecting the sub fertile male. A short description is given for each below.

4.2.1 Reactive oxygen species (ROS)

a) Global seminal ROS

Global ROS, i.e. measure both extra- and intra-cellular ROS by chemiluminescence assay using a luminal or lucigenin probes. Luminol is more sensitive and reacts with a variety of ROS at neutral pH. Free-radicals oxidize luminol/ lucigenin to produce a luminescence signal which is converted to an electrical signal by the luminometer. Level of free radicals produced is measured as relative light unit per second (RLU/sec) per million sperm [79].

b) Specific intra-cellular ROS

Intracellular generation of ROS can originate from a specific defect in number of potential sources, including NADPH oxidase and complex III within the mitochondrial electron transport chain. Dichlorofluorescin (DCF) can detect intra-cellular H_2O_2 and dihydroethidium (DHE) can detect intracellular O_2 [80]. Mean fluorescence intensity and percentage of positivity will be reported for each specimen.

Figure 2 **(A)** Routine sperm parameters in healthy (Right) and infertile (Left); Opacity with pH and ejaculate volume in the same time, Sperm morphology by diff quick staining method, Sperm viability stained with one step Eosin-Negrosin method, Cytospin preparation stained with Nuclear-Fast Red and picroindigocarmine staining (NF-PICS) that stain sperm heads in red. **(B)** Sperm Chromatin cytochemical staining developed earlier such as acridine orange test, Aniline blue test, and toluidine blue assay. Comet assay with either neutral or alkaline buffer, 2-tail Comet assay when both alkaline and neutral buffers used. Flowcytometry based assays were developed to assess sperm DNA fragmentations as

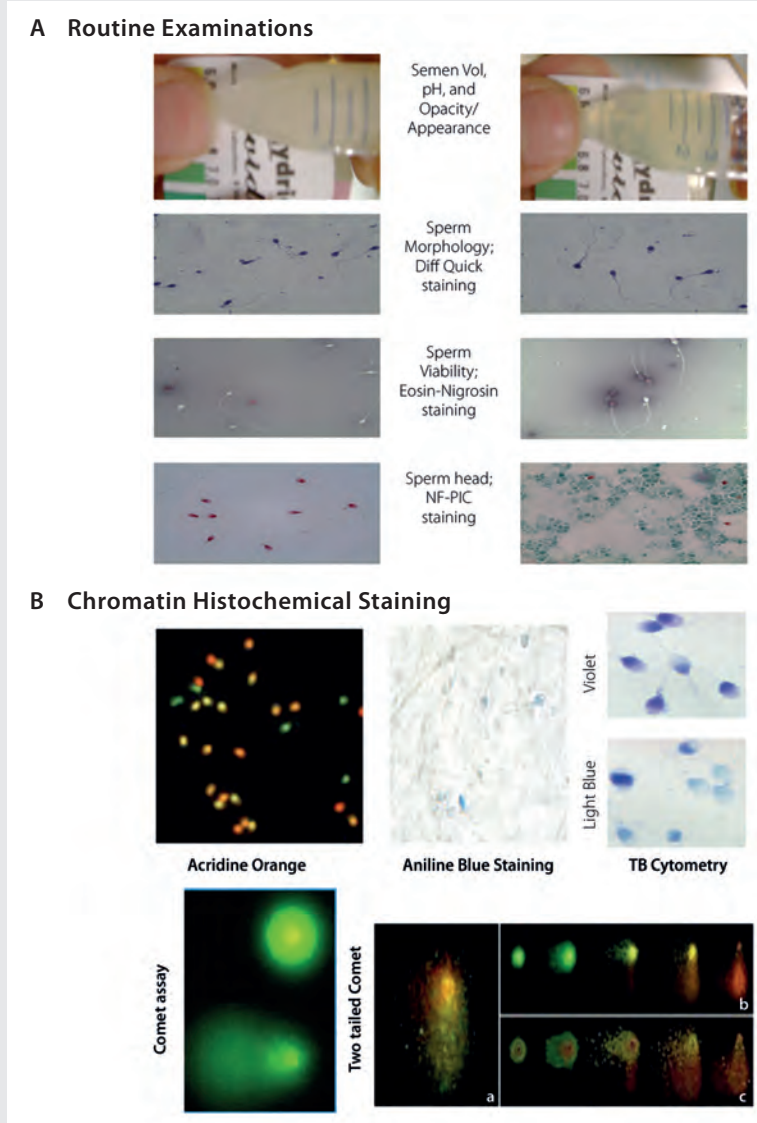
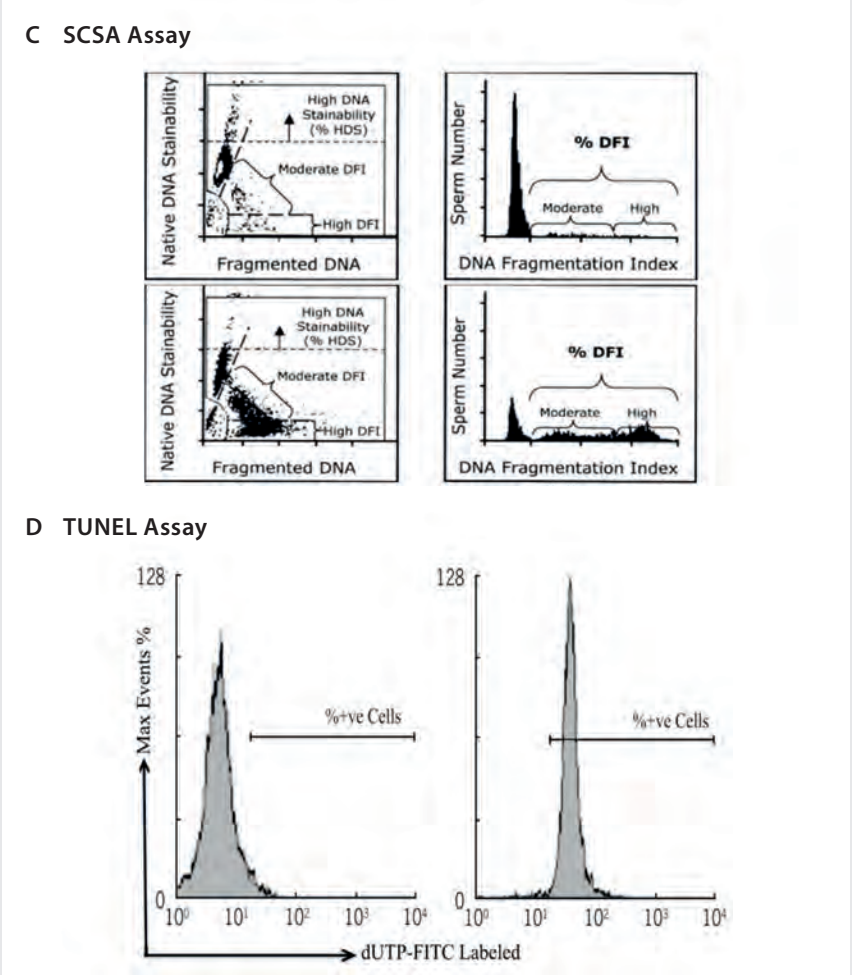


Figure 2 **(C)** sperm chromatin structure assay (SCSA®) and **(D)** Terminal deoxynucleotidyl transferase dUTP nick end labeling (*TUNEL*) assay.



4.2.2 Seminal plasma antioxidant capacity (TAC)

Detection of seminal plasma available TAC is an indirect assay for OS and may provide the clue for sub fertile male diagnosis and management [81].

4.2.3 Lipid perioxidation

OS can have major detrimental effects on sperm lipid membranes, which are rich in polyunsaturated fatty acids. Thus, by increasing the level of membrane lipid perioxidation, they diminish the membrane fluidity and its functions. These peroxides decompose the Fe(2+)-promoted thiobarbituric acid (TBA) assay, stimulating a lipo-peroxidative chain reaction and resulting in malondialdehyde formation [82].

4.2.4 Sperm Integrity

The assumption is that defective sperm chromatin (mainly due to DNA breaks) will impair embryo development and implantation [83]. Assays available to measure directly the sperm DNA damage are e.g. TUNEL in situ nick labeling and Comet (Figure 2 B and D). Indirectly measurements of DNA damage are aniline blue, toluidine blue (TB), SCSA®, CMA3, and halo test among others (Figure 2 B, C and D). Some data showed negative effect on fertilization and IUI success in samples with poor sperm quality [33] but no clear negative impact on implantation with IVF or ICSI in males who had DNA fragmentation index (%DFI) of > 30% and increased miscarriage rate [84]. All these assays may provide different prediction levels for abnormal sperm chromatin or sperm DNA fragmentation (**Figure** 2-C to 2-D). However, they are still in need of more standardization.

a) Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL)

This assay is used to detect fragmented DNA which utilizes a reaction catalyzed by an exogenous TdT and is termed as ''end labeling'' or terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. It has been reported to be a specific and reproducible test for sperm DNA damage detection [85](Figure 2-D).

b) Sperm Chromatin Structure assay (SCSA®)

SCSA is a flow-cytometric assay based upon the fact that abnormal sperm chromatin is highly susceptible to physical induction of partial DNA denaturation in situ [86]. The extent of DNA fragmentation index (DFI) following acid treatment is determined by measuring the metachromatic shift from green fluorescence (acridine orange intercalated into double-stranded nucleic acid) to red fluorescence (acridine orange associated with single-stranded DNA) [87]. Mean green fluorescence reflects DNA content and/or degree of sperm chromatin condensation, of which the latter inhibits DNA stainability. Percentage of high DNA-stainable (%HDS) sperm identifies the cells with immature nuclei due to lack of nuclear condensation [88] (**Figure** 2-C).

c) Sperm Chromatin Dispersion (SCD) Test

SCD assay has the ability to diagnose different degrees of DNA fragmentation with severe damage being characterized as degraded DNA. SCD test is based on the principle that sperm with fragmented DNA fail to produce the characteristic halo when fixed in low melting agarose microgel then acid denaturation and removal of nuclear proteins [89]**.**

Sperm with intact DNA form chromatin loops (halo). DNA strand breaks prevent this looping; the degree of DNA damage is proportional to the decreasing size of the loops ranging from normal big and medium halos down to mild or no halo. Degraded cells are counted for calculation of DNA degradation index (the nuclear core).

d) Comet Assay

The single cell gel electrophoretic assay (Comet assay) was applied for sperm [90]. This method may be done with high alkaline conditions (pH 12.5) that denatures all DNA or with neutral pH (not causing DNA denaturation). Two tails comet assay was proposed for detecting single and double stranded DNA breaks simultaneously [91] (**Figure** 2-B).

e) Chromomycin A3 (CMA3)

CMA3 is a glycosidic anti-neoplastic antibiotic isolated from the bacterium Streptomyces griseus. CMA3 reversibly binds to guanine-cytosine (G-C) base pairs in the minor groove of DNA. This agent is used as a fluorescent chromosome dye. The binding of CMA3 is an indication of poor chromatin condensation [92](**Figure** 2-B).

f) Toluidine Blue (TB) staining

TB staining was described as an alternative to sperm chromatin structure assay. TB staining positivity can be categorized as: normal (light blue, LB %), abnormal (dark violet, DV %) and two intermediate chromatin conformations (blue, B %; and Violet, V %) [93](**Figure** 2-B).

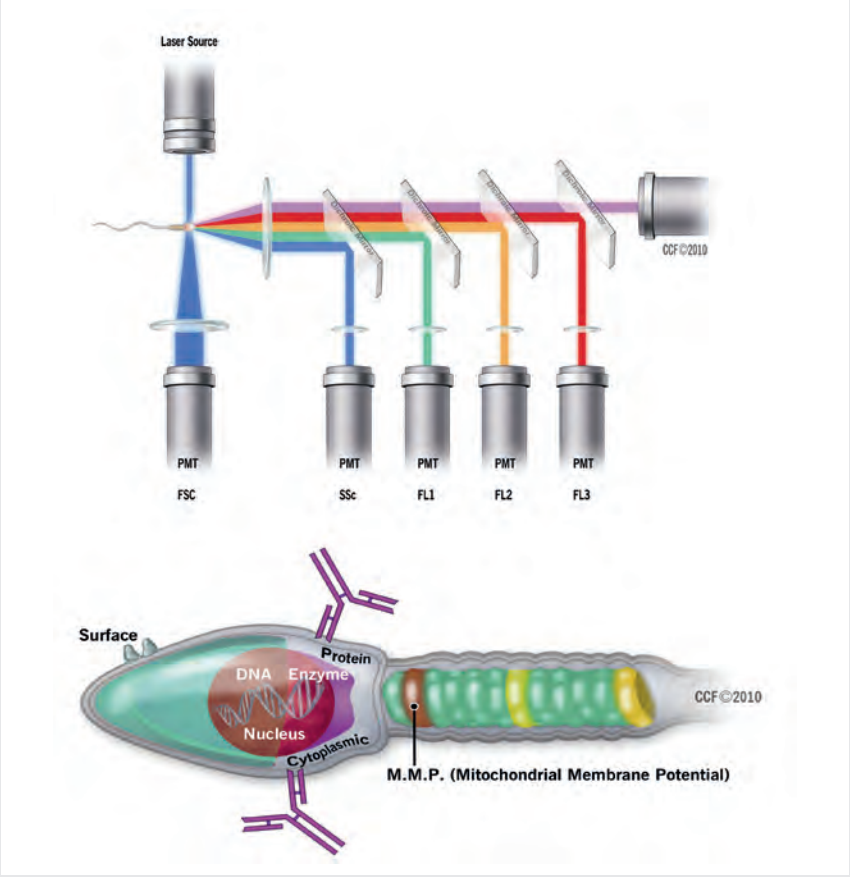
4.3 Suggested Techniques for the future Reproductive Laboratory

4.3.1 Flow Cytometry (FCM)/ Cytometrics (Figures 2-C and D)

Accurate detection of a sperm marker depends on the ability of FCM to precisely orient the spermatozoa at the time of measurement in the flow cell of the analyzer or sorter. The sperm wave generated after exposure to the laser beam shows a characteristic pattern due to the presence of the tail following the head for each spermatozoon. Sperm may be stained by single or multiple markers for accurate FCM detection of sperm characteristics (Figure 3). The staining quality for each sperm compartment can be examined by flowcytometry and should always be verified by a fluorescent microscope [94].

Sperm membrane, cytoplasmic or nuclear markers can be examined by the flowcytometry (Figure 3). These assays may be used for diagnostic and/ therapeutic purposes. Sperm sorting can be based upon markers such as surface markers e.g. membrane damage by using annexin V fluorescence labeled antibodies or intracellular markers such as DNA contents [95, 96]. New FCM assays were proposed to play a major role for detection of sperm pathologies [97]. Apoptosis detection methods should be analyzed in light described recently as well as sperm gating [98].

Figure 3 Flowcytometry (FCM) is a rapidly growing technology in andrology lab. **(Upper part)** FCM is basically composed of laser source that hits a single cell at one time (sheath fluid hydro focusing, fluidics). The reflecting signals are collected through a series of dichroic mirrors and filters (Optics) and amplified by photomultiplier tubes (PMT) to provide a digital information on physical (FSc, forward scatter), granularity (SSc, side scatter) and chemical cellular properties (fluorescent light, FL). Multiple sperm properties and markers can be examined through surface, intra-cytoplasmic and/ or nuclear staining of spermatozoa (**Lower part**).



4.3.2 Molecular Biological Based Techniques

a) Proteomics

Proteomics technologies have improved its sensitivity in protein detection in matured sperm and it has therefore driven a better insight in the normal proteome [99]. The key challenge is to move from lists of identified human sperm proteome to informed understanding of biological function or application [100].

b) Metabolomics

Metabolomic is also an emerging technique for the profiling key biomarkers of living cells. This is a promising assay that can be beneficial in the management of male-factor infertility and ART in general. Metabolomics is a non invasive analysis in the investigation of infertile men. Unique metabolomic profile of oxidative stress biomarkers (-CH, -NH, -OH and ROH) has been reported to be of characteristic pattern using semen plasma from healthy men vs. patients with idiopathic infertility, varicocele and vasectomy reversal [101].

c) Genomics

Observations have demonstrated that the environmental agent has the ability to induce genetic and epigenetic changes in the sperm genome. Such changes might induce trans-generational changes, expressed in adults onset to diseases [102]. Epigenetic modifications, gene sequences and gene expression may play a growing role in male infertility and reproduction laboratories although they still require more standardization [99, 103].

Scope of the thesis

The current pregnancy and live-birth rates in ART remain to be improved. Continuous efforts are designed to increase the likelihood that a couple undergoing IVF will have a healthy baby. The increase in andrology applications associated with suboptimal success rates has mandated the development of a laboratory approach for a more accurately diagnose in order to protect the quality of gametes and their resulting embryos after fertilization. A clinical pathology approach includes evaluation of semen samples by the most common laboratory techniques, and introducing novels assays which cover examination of additional markers in gametes that could be used in ART applications. While the role of spermatozoa in ART failure has been overlooked, strong evidence suggests that proper use of healthy spermatozoa will result in improvement of ART success rates.

Aim of the thesis

In this thesis, our aims were to: 1) examine the role of OS in male infertility, specifically intracellular ROS in the occurrence of spermatozoa-mediated damage with apoptotic features which compromise male fertility potential; 2) examine the clinical value of novel markers for diagnosis and 3) apply these novel markers on clinical setting for male factor management.

Our main motivation is to develop new protocols for clinico-pathological diagnosis based on OS or apoptosis-like damage markers and manifestations. Such an approach represents the future of laboratory diagnostics that expands to include molecular characterization of spermatozoa in addition to the routine examination.

Outline of the thesis

This doctoral thesis consists of three main parts:

Part I: Sperm maturity, OS and apoptosis

Spermatogenesis is controlled by combined genetic and microenvironmental effects. Sperm maturation requires physiological levels of ROS. Defective spermatogenesis may be related to OS and apoptosis. Immaturity may be associated with OS and apoptotic changes which affect the quality of male gametes (reviewed in the general introduction, **Chapter 1**). It is difficult to define healthy mature spermatozoa only on basis of routine sperm parameters in infertile men. There is an increased need for novel markers and accurate tools for optimization of diagnostic or prognostic services for andrology laboratory.

Part II: Novel markers for OS and apoptosis

This part includes studies which cover evaluation, adaptation and validation of novel assays of OS damage and apoptosis which can be used to characterize (healthy) spermatozoa along with routine sperm parameters. Recently, expression of poly (ADP-ribose) polymerase (PARP) homologues has been reported to differ with fertility potential as well as with sperm maturation. In **chapter 2** we reviewed any potential biological role of of this novel marker, PARP-1, the most important protein in DNA damage repair, in male gametes. In **chapter 3**: we examined PARP cleavage (cPARP) in ejaculated human spermatozoa as an earlier marker for DNA damage especially after OS- or chemical- induced sperm stresses. In **chapter 4**: we examined the incidence of a single nucleotide polymorphism (SNP) of endothelial nitric oxide synthase (eNOS) gene (Glu298asp variant) in infertile men with asthenozoospermia. Idiopathic asthenozoospermia may carry a genetic defects of certain enzymes involved in free radicals metabolism. **Chapter 5**: we have examined the relationship of sperm viability, apoptosis, with intracellular ROS levels in human spermatozoa within immature and mature sperm fractions. We aimed also to examine the effect of OS on sperm viability with high levels of intracellular ROS.

Part III: Applications of Apoptotic Markers in Andrology

In this section we applied our in-vitro studies to clinical settings. In **chapter 6** we studied the diagnostic value of TAC in human seminal plasma. We used receiver

operating curve (ROC) analysis for determining cutoff values and ranges of seminal plasma TAC in infertile and healthy donors. In **chapter 7**, in a comparative study, we have evaluated flowcytometry and chemiluminescence as measures for ROS in human semen. We compared the detection limitations of both methods in a pre-clinical study for assay development. In **chapter 8**: we studied semen characteristics and sperm DNA fragmentation in infertile men with low and high levels of seminal ROS. We examined the possibility of the presence of extensive sperm DNA fragmentation with high ROS levels in infertile men. In **chapter 9**; we demonstrated the relationship of seminal ROS levels with sperm concentration, motility and abstinence time. In a longitudinal study, we followed up sperm concentration, total motile sperm and seminal ROS over 21 months in a normozoospermic sperm donor. We aimed to examine any fluctuation in these parameters over time.
Finally, the findings of this thesis are discussed **chapter 10**.

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Part II

Novel Markers for OS and Apoptosis

Chapter 2

Potential biological role of poly (ADP-ribose) polymerase (PARP) in male gametes

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Abstract

Maintaining the integrity of sperm DNA is vital to reproduction and male fertility. Sperm contain a number of molecules and pathways for the repair of base excision, base mismatches and DNA strand breaks. The presence of Poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme, and its homologues has recently been shown in male germ cells, specifically during stage VII of spermatogenesis. High PARP expression has been reported in mature spermatozoa and in proven fertile men. Whenever there are strand breaks in sperm DNA due to oxidative stress, chromatin remodeling or cell death, PARP is activated. However, the cleavage of PARP by caspase-3 inactivates it and inhibits PARP's DNA-repairing abilities. Therefore, cleaved PARP (cPARP) may be considered a marker of apoptosis. The presence of higher levels of cPARP in sperm of infertile men adds a new proof for the correlation between apoptosis and male infertility. This review describes the possible biological significance of PARP in mammalian cells with the focus on male reproduction. The review elaborates on the role played by PARP during spermatogenesis, maturation, ejaculated spermatozoa and the potential role of PARP as new marker of sperm damage. PARP could provide new strategies to preserve fertility in cancer patients subjected to genotoxic stresses and may be a key to better male reproductive health.

Keywords: PARP, infertility, spermatozoa, spermatogenesis, oxidative stress, DNA Repair, apoptosis, PARG.

Introduction

Male fertility is affected by a variety of environmental, behavioral, and genetic factors that can alter spermatogenesis at various levels [1-3]. Male germ cells are exposed to a wide variety of endogenous and exogenous genotoxic agents. Endogenous agents include reactive oxygen and nitrogen species generated during the metabolic activities of cells [4, 5]. Exogenous agents include various environmental factors that can inflict damage to genomic DNA. These genotoxic agents can introduce DNA lesions in the form of DNA single and double strand breaks, abasic sites, base damage, inter-and intra-strand cross links and DNA-protein cross links [6, 7]. Origin of DNA damage in human spermatozoa can occur by abortive apoptosis, abnormal chromatin packaging, generation of reactive oxygen species and premature release from Sertoli cells [8-12]. During spermatogenesis, germ cell nucleus is nicked by topoisomerases in order to relieve the torsional stresses created when DNA is compacted into the differentiating sperm head. Persistence of DNA strand breaks during different stages of spermatogenesis contribute to DNA damage detected in mature spermatozoa [13, 14]. As spermatids are haploid, they must resolve double stranded DNA breaks by an error-prone DNA repair mechanism [15]. An interest in male germ cell DNA quality has increased in recent decades especially in the era of assisted reproductive technologies (ART). As natural selection of spermatozoa for fertilization is bypassed in procedures such as intracytoplasmic sperm injection (ICSI), awareness has been raised regarding the possibility of congenital anomalies. Many reviews have dealt with the origin of sperm DNA integrity, evaluation of available technologies to assess sperm DNA integrity and its impact on the ART outcome [16-24]. Environmental, life style and occupational hazards in male infertility have also been extensively studied [25-33]. These factors may affect DNA repair pathways and impact male fertility and subsequent embryo development.

In this review we will discuss the role of Poly (ADP-ribose) polymerase (PARP) as one of the DNA damage repair proteins and highlight its role in ejaculated human spermatozoa. Recently the focus of PARP's role in malignancy has intensified to include use of PARP inhibition as an adjuvant therapy with chemotherapeutic drugs [34]. As our interest lies primarily in male reproductive health, in this review we will focus on the biological role of PARP in general as well as in male gamete and highlight possible role of PARP in modulating DNA damage in male germ cells.

Poly (ADP-ribose) polymerase (PARP)

Poly (ADP-ribose) polymerase (PARP), a nuclear enzyme has a particularly well-researched role in base excision repair; it is one of the primary repair mechanisms to resolve DNA lesions caused by endogenous processes as well as those caused by exogenous chemical exposure and irradiation [35, 36]. PARP also has a well-documented

role in testicular germ cells [37-39], including a role in DNA damage repair of germ cells [40]. However, a similar role for PARP in human ejaculated spermatozoa is still being investigated. The last decade has seen increasing interest in the relationship between DNA integrity in mature ejaculated spermatozoa and male infertility [16, 25, 41-43]. Focus on the genomic integrity of the male gametes has been further intensified by the growing concern about the transmission of genetic diseases through intracytoplasmic sperm injection (ICSI) [44-49].

Proteins involved in the major repair pathways have been shown to be expressed in the testis [50]. PARP proteins are involved in detection of strand breaks and signaling in both the base excision repair and nucleotide repair pathways [51, 52]. PARP catalyzes poly (ADP-ribose) (PAR) polymerization from donor NAD^+ molecules into target proteins. PARP1 is the prototype and most abundantly expressed member of a family of PARPs. The PARP family consists of 18 homologues (PARP 1-18) with a conserved catalytic domain made up of 50 amino acid residues that serve as the 'PARP signature' [53]. This is the site where poly(ADP-ribose) (PAR) chains are initiated, elongated, and where branching of the chains can occur [54]. Besides this catalytic domain, PARP family members may also have other domains including DNA binding domains, macro-domains, breast cancer-1 (BRCA-1) C-Terminus (C-T) domain, ankyrin repeats and a domain associated with protein-protein interaction called WWE. BRCA-1 C-T domains are characteristic of proteins responding to DNA damage at cell cycle checkpoints while WWE domains are found in proteins associated with ubiquitination. All of these special types of domains contribute to the unique functions of each family member [53, 55, 56].

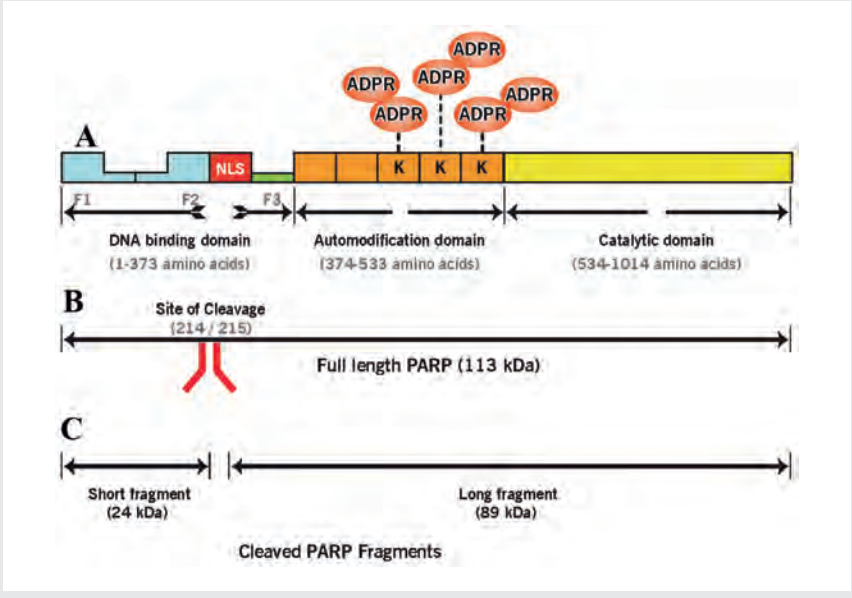
PARP family members can be divided into several subcategories or groups based on each protein's established functional domains and precise functions into: 1) *DNA dependent PARPs* (PARP1 and PARP2) that are activated by DNA strand breaks 2) *Tankyrases* (tankyrase-1 and tankyrase-2) that serve diverse functions such as telomere regulation and mitotic segregation 3) *CCCH-type PARPs* (PARP12, PARP13) which contain special CCCH type zinc fingers and 4) PARP9, PARP14, and PARP15 consisting of macro PARP's which have 1-3 macrodomains connected to a PARP domain. They also have WWE domain and PARP catalytic activity. PARP6, 8, 11 and 16 do not have any recognized domains or functions and therefore they have not been assigned proper nomenclature [56].

Recent classification system by Hassa and Hottiger groups PARPs on the basis of their catalytic domain sequences[54]. PARP family is divided into 3 separate groups: 1) PARP1, PARPb (short PARP1), PARP2, and PARP3, 2) PARP4 and 3) 2 PARP members, Tankyrase-1, tankyrase-2a, and its isoform tankyrase-2b (also known as PARP5 and PARP6a/ b) [54]. The various PARP enzymes can also have different subcellular localization patterns. PARP1 and 2 are considered nuclear enzymes and are found in the nucleus of cells. In contrast, tankyrases and PARP3 are found in both the nucleus and cytoplasm [54, 57].

Perhaps the best studied member of the PARP family is PARP1, a 113 kD enzyme encoded by the ADP-ribosyl transferase (ADPRT) gene in humans located on chromosome 1 [58, 59].

PARP1 has been reported to be involved in regulation of chromatin structure and transcription processes in response to specific signaling pathways [55]. The protein structure of PARP1 is well characterized. Figure 1 represents the PARP1 structure domains with clarification on the sites of the zinc fingers, PAR acceptance, and cleavage site with short and long cleaved PARP1 fragments. PARP1 is made up of 3 functional domains including DNA binding domain (DBD), automodification domain (AMD) and catalytic domains (CD). The DNA binding domain contains zinc fingers that can bind to breaks in DNA and contains the nuclear localization signal (NLS), which ensures the translocation of PARP1 into the nucleus and also forms a site of cleavage by caspase 3. The AMD is responsible for addition of ADP-ribose polymers to PARP1 itself. The catalytic domain is responsible for the PARP activity [60] (Figure 1).

Figure 1 Structural domians of PARP and its fragments showing **A**: DNA binding domain containing Zinc fingers (F1-F3) for nucleosome binding and nuclear localization (NLS) segment; Automodification domain responsible for adding ADPR (ADP-ribose) polymers through binding with Lysine (K) amino acid and catalytic domain has the PARP signature and PARP enzymatic activity. **B**: Full length PARP1 113 KDa molecule with a mark on the site of cleavage (214/215 amino acids) **C**: PARP cleavage by caspase showing short (24 KDa) and long (89 KDa) cleaved PARP fragments.



The DNA binding domain extends from the initiator methionine (M) to threonine (T) 373 in human PARP1, and contains 2 well known structurally and functionally unique zinc fingers (FI: amino acid 11–89; FII: amino acids 115–199) [61, 62]. A recently discovered third and thus far an unrecognized zinc-binding motif, (FIII: amino acid 233–373) has been reported [63, 64]. DNA binding domain contains a bipartite nuclear localization signal (NLS) of the lysine (K) rich form KRK-X -KKKSKK (amino acid 207–226) that targets PARP1 to the nucleus [65]. Zinc fingers FI and FII are thought to recognize altered structures in DNA rather than particular sequences. These zinc fingers have been reported to be involved in protein – protein interactions [66]. PARP1 strongly associates with single and double strand DNA breaks generated either directly by DNA damage or indirectly by the enzymatic excision of damaged bases during DNA repair processes. Several studies suggest that the first zinc finger is required for PARP1 activation by both DNA single and double strand breaks, whereas the second zinc finger may exclusively act as a DNA single strand break sensor [61, 62, 67]. Additional studies are necessary for further identification of interactions/localization of PARP or different mutation/ polymorphisms of PARP in the pathophysiology of oxidative stress, apoptosis and malignancies [68].

The major PAR acceptor protein is PARP1 itself, which appears to accumulate roughly 90% of cellular PAR via PARylation of its auto-modification domain. Binding of NAD (monomer of ADP-ribose) with PARP1 is mainly through hydrogen bonds and other with the oxygen molecule from glutamic acid residue on the c-terminus mainly the automodification domain [53]. Interestingly, Altmeyer et al (2009) showed that glutamic acid residue in the automodification domain of PARP1 is not required for PAR formation. Instead they identified lysine residues to be the PAR acceptor sites in PARP1[67].

Role of PARP in metabolic pathways

The real challenge and the difficulty in understanding PARP interaction and PAR metabolism is the lack of structural information that can be provided by X-ray crystallography or by nuclear magnetic resonance (NMR). Prior to the recent findings by Altmeyer et al [67], the intense research on PARP was unable to confirm the presence of glutamic acid residue in the AMD that may be functioning as the PAR acceptor of amino acid in PARP1 [69]. This was mainly due to the lack of mutational studies for PARP. Figure 2 explains the possible PARP interaction with the main proteins involved in DNA repair during DNA damage/ repair process. The primary catalytic function of PARP enzymes is to transfer ADP-ribose groups to the glutamate, aspartate and carboxy-terminal lysine residues of proteins. With NAD serving as a cofactor, PAR polymerization begins by breaking the glycosidic bond between ADP-ribose and nicontinamide. PARP can elongate the amino acid chains of recipient proteins in a linear or branched manner by the addition of up to 200 ADP-ribose groups [70].

PARP attaches ADP-ribose groups to a variety of protein substrates. Perhaps the most common target of (ADP-ribosyl)ation is PARP1 itself, termed auto-modification [71]. PARP enzymes commonly modify nucleosome proteins in order to restructure chromatin. While both histone H1 and H2B are acceptors of poly ADP-ribose, histone H1 is the major acceptor recipient [72, 73]. Histone H1t, a major H1 variant in testis, is the main H1 target of PARP during spermatogenesis [74]. PARP activity is not restricted to nuclesome proteins; the well-known tumor suppressor protein p53 is also modified by PARP1. This modification transcriptionally inactivates p53 [75]. DNA polymerases modified by PARP have also been inhibited during *in vitro* studies [76]. Toposiomerase II can also be modified by PARP activity [77]. Similarly, the transcription nuclear factor kappa β (NFk-β) can also be modified by ADP-ribose group attachment [78].

Regulation of PARP activity is important for exploring the therapeutic options of this enzyme. Several types of molecules have been identified as activators of PARP activity including histones, a common target of PARP. Though histones H1 and H2B are modified by PARP1, histones H1 and H3 reciprocally activate PARP1 [79, 80]. Apart from ribosylation, the structure of histones is regulated by acetylation and silent information regulator gene (SIRT1), a histone deacetylase, involved in the maintenance of histone structure. SIRT1 has a regulatory action on PARP1 activity and in the absence of SIRT-1, PARP remains unregulated resulting in apoptosis inducing factor (AIF) regulated cell death [81]. PARP activity is also activated by a number of metal ions (like magnesium and calcium) and polyamines. Incidentally, calcium ions also play an important role in the pathophysiology associated with oxidative stress and could provide a link to explain the effect of oxidative stress on PARP activity [82-86].

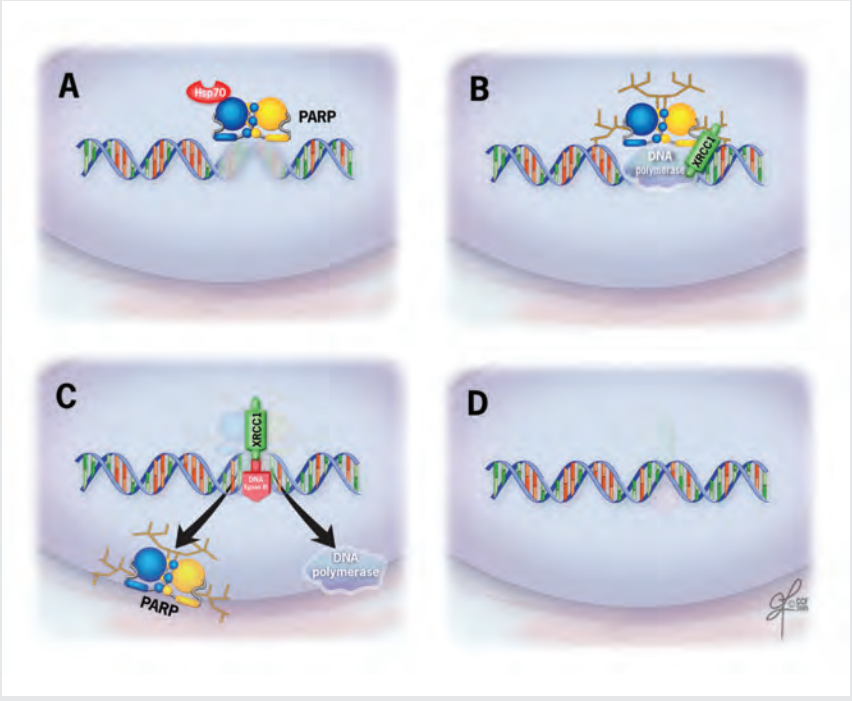
There are a number of inhibitors used to study PARP activity such as endogenous purines (hypoxanthine and inosine) or exogenous molecules like caffeine derivatives or tetracycline derivatives [80, 87]. PARP1 phosphorylation by ERK1/2 is required for maximal PARP1 activation after DNA damage [88]. Furthermore DNA-dependent protein kinase (DNA-PK), a protein involved in the repair of double strand breaks results in suppression of PARP activity probably through direct binding and/or sequestration of DNA-ends which serve as an important stimulator for both DNA-PK and PARP [89].

PARP in mammalian cells

The most important role of PARP is its capacity to repair DNA, especially in resolving single strand breaks. PARP1 and PARP2 have been shown to function in the repair of base excision [60]. Additionally, PARP1 may play a role in an alternate version of double strand DNA break repair involving the DNA repair protein XRCC1 (X-ray repair complementing defective repair in Chinese hamster cells 1) along with Ligase III and DNA-dependent protein kinase involved in resealing DNA breaks [90, 91]. PARP interactions and its role in the DNA repair process is explained in Figure 2. PARP1/ PARP2 get activated with DNA breaks and interact along with other main DNA repair proteins

(XRCC1, DNA polymerase, DNA ligase III, and other DNA binding proteins to repair the damaged DNA strand. Heat shock protein may provide additional activation for the PAR formation early in the DNA repair [92] (Figure 2).

Figure 2 PARP interactions in DNA damage/ repair showing **A**: DNA damage caused by genotoxic agents activates PARP with HSP70 (heat shock protein 70) providing further activation **B**: activation of PAR formation with further help from other DNA repair proteins such as XCCR1 (X-ray repair complementing defective repair in Chinese hamster cells) that help DNA polymerase to start sealing the damaged DNA strand **C**: DNA polymerase and ligase seal the DNA nicks releasing PARP and other DNA binding proteins and **D**: repaired DNA.



PARP1 binds to broken strands of DNA and automodifies itself. It then dissociates from the single DNA strand due to the negative charge it acquires from the ADP-ribose group. After dissociating, PARP associates with DNA Ligase III alpha, which is involved in the resealing of DNA breaks, and XRCC1. XRCC1 then recruits other repair factors (such as DNA polymerase β, apurinic-apyrimidinic (AP) endonuclease, and polynucleotide kinase) to complete the repair process [93]. Thus, PARP1 is part of an important signaling pathway for DNA damage repair due to its ability to recruit other

repair enzymes necessary to preserve DNA integrity of a cell. The role of PARP1 in base excision repair (BER) was recognized by its interaction with DNA polymerase β and its interaction with DNA Ligase III and XRCC1. It was also shown that PARP1 deficient cells demonstrated significantly inhibited BER activity [94].

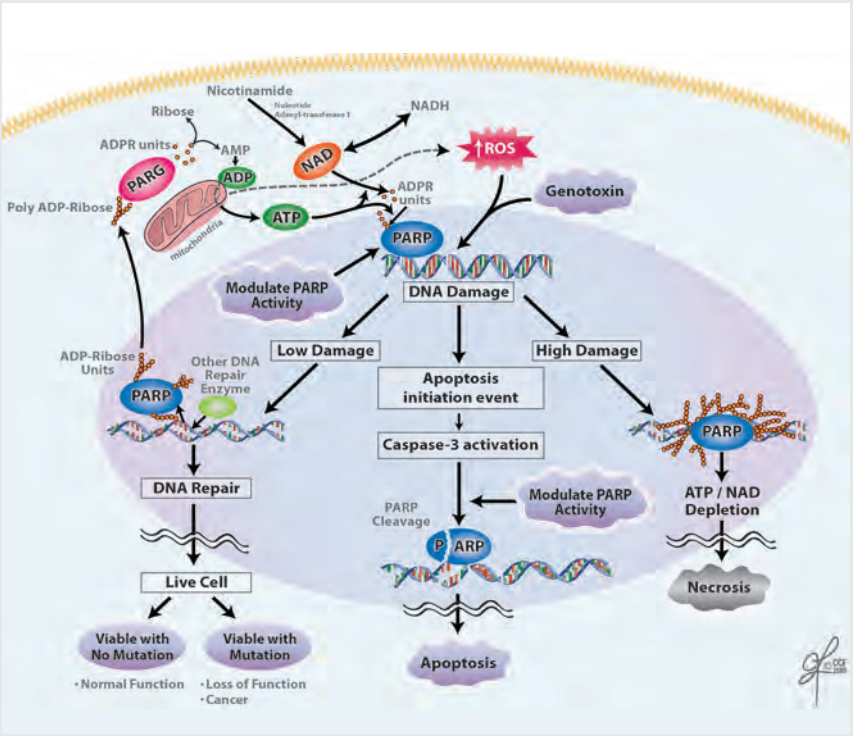
Similarly, PARP2 has also been shown to participate in BER pathways, associating with DNA polymerase β, XRCC1, and DNA Ligase III [51]. PARP2 deficient mice showed significant delays in resealing single strand DNA breaks similar to those seen in PARP1 deficient mice. This is interesting because PARP2 activity is 10 times less than that of PARP1. PARP1 and PARP2 also appear to homodimerize and heterodimerize as a part of DNA repair [51]. PARP not only serves as part of a signaling pathway in DNA damage repair but it is also involved in the repair process. In particular, the binding of PARP1 to the broken ends of DNA may protect it from degradation by nucleases [95]. NBS1 (Nijmegen Breakage Syndrome 1) has been recently reported to be required for base excision repair (BER) [96].

A model put forth recently suggests that the involvement of PARP in DNA damage repair is regulated by a feedback mechanism [97]. PARP1 is first recruited to the DNA break site and then binds to this site through its DNA binding domain. The addition of poly (ADP-ribose) (PAR) units enables the recruitment of more PARP1 moieties through the AMD of PARP1. The aggregation of PARP molecules then creates a signal that recruits other repair factors and forms the positive feedback mechanism of PARP. Interestingly, PARP activity is subject to a negative feedback mechanism that prevents excessive accumulation of poly (ADP-ribose) and cell death [98, 99].

PARP in cell cycle and Cell death

In addition to being involved in the rescuing function of DNA repair, PARP1 is also directly involved [100] in both programmed cell death as well as necrosis [101-103] (Figure 3). PARP is part of the caspase-dependent pathway of apoptosis and as part of this caspase mediating pathway; PARP1 is cleaved by Caspase-3 into a 25 kDa N-terminal and an 85 kDa C-terminal fragment. The 25 kDa fragment consists of the DBD and the 85 kDa fragment consists of the AMD and CD (Figure 1). The detachment of the DNA-binding domain from the automodification and catalytic domains inactivates PARP1 and allows apoptosis to occur [104]. Meanwhile, the N-terminal fragment inhibits any uncleaved PARP1 molecules that are still present. Thus, PARP1 cleavage effectively inhibits PARP activity that could result in energy depletion as a result of consumption of a significantly large number of Nicotinamide adenine dinucleotide (NAD) molecules and lack of ribosylation [105]. PARP1 is also involved in a caspase-independent cell death pathway. When PARP1 is activated by exposing fibroblast cultures to hydrogen peroxide-induced damage, it triggers caspase-independent pathway. PARP activation triggered the release of AIF from the mitochondria and caused it to relocate to the nucleus resulting in chromatinolyis [55, 106-108].

Figure 3 Possible role of PARP in cell death in the event of DNA damage caused by ROS or a genotoxin, PARP targets the damaged site. If high damage occurs, PARP may become over-activated resulting in ATP/ NAD depletion and necrosis. Apoptosis can also occur through caspase-3 activation and PARP cleavage. If low damage occurs, PARP can recruit other repair enzymes and DNA repair can occur. Recently PARP1 dependent cell death termed as parthanatos has been reported, and is distinct from apoptosis, necrosis or autophagy.



PARP is also involved in a less organized version of cell death-necrosis. Originally Berger et al proposed that depletion of NAD caused an over-activation of PARP and in the milieu of excessive DNA damage can cause necrosis [109]. Heeres and Hergenrother proposed that over-accumulation of poly (ADP-ribose) are cytotoxic to cells and induce them to undergo necrosis. Thus PARP appears to be a two-sided coin. It has the potential to respond to threats to DNA integrity, but its over-activation can lead to cell death [101]. Figure 3 represent a schematic pathways link the ROS and genotoxins induced DNA damage with the ATP/NAD levels that may determine the cell death pathway depending upon the extent of the DNA damage, caspase activation, and ATP/ NAD levels. Modification of PARP activity through inhibition or cleavage may lead to apoptosis through preventing DNA repair provided by PARP. However, recent

report indicate that PARP may lead to PARP1 dependent cell death that is reported to be distinct from apoptosis, necrosis or autophagy and it is called parthanatos [110].
The role of PARP in maintaining genomic integrity could have profound implications for cell division. PARP is associated with specific structures important for cell division such as centromeres and centrosomes, pointing to its possible role in cell division. PARP1 associates with centrosomes during cell division and interphase, which may be related to PARP1's role in maintaining chromosome stability [111]. PARP1 is also involved in spindle structures necessary for cell division. Poly (ADP-ribosyl)ation of spindle structures is essential for normal functioning and assembly of spindles that if disturbed could result in defective chromosome segregation [112, 113]. PARP3 also demonstrated an interaction with PARP1 at centrosomes [57]. PARPs also localize to active mammalian centromeres primarily during metaphase and prometaphase [114, 115]. PARP1 and PARP2 bind to the same centromere proteins, including the important mitotic checkpoint protein and Bub3 (budding uninhibited by benzimidazoles 3) [55].

Chromatin remodeling and transcription

Poly(ADP-ribosyl)ation can be considered an epigenetic modification [116] because its modification of histones can remodel chromatin structure in order to provide unique information for other proteins involved in not only DNA repair but also in transcription [55, 97].
The role of PARP in transcription is a result of two important aspects of its function: its modification of histones and interaction with other coactivators and DNA binding factors to bind to the enhancer promoter regions [117-119].
The chromatin environment is very important for transcription and the role of PARPs in chromatin remodeling has been well documented [55, 108]. PARP has been found to associate with the Facilitates of Chromatin Transcription (FACT) complex, a protein complex involved in chromatin remodeling. Poly (ADP-ribosyl)ation of the FACT complex prevents the interaction between FACT and nucleosomes *in vitro* [120]. As mentioned before, Histone H1 and H2B are major acceptors of PARP modification and this modification can also remodel chromatin structure [116]. Specifically poly (ADP-ribosyl) ation of nucleosome structures can cause relaxation of chromatin structure [116, 121, 122].
In a recent study, PARP1 was shown to be broadly distributed across the human genome. Furthermore, PARP1 and histone H1 were shown to have reciprocal roles in regulating gene expression. When there is increased presence of PARP and decreased presence of H1 at promoter regions of genes, the genes are activated in 90% of the cases. When gene promoters had both decreased presence of PARP and H1, less than 45% of genes were expressed [123]. Tulin and Spalding reported that PARP can activate the transcription of heat shock proteins in Drosophila by decondensing chromatin structure [108]. This was in contradiction to the study of Oei et al who showed that poly (ADP-ribosyl)ation of transcription factors for TATA-binding protein (TBP) or YinYang1 (YY1) prevented these transcription factors from binding DNA [124]. However, once TBP

or YY1 were bound to DNA, they were immune to the action of PARP. Therefore, PARP cannot dislodge TBP or YY1 once they are bound to DNA. In this way, PARP can prevent transcription in specific parts of the genome without disturbing ongoing transcription [60, 125, 126].

PARP interacts with transcription factors at enhancer and promoter regions via its involvement with NF-κβ, [23, 78, 127, 128]. PAPR-NF-κβ interactions have important consequences for inflammation. PARP1 deficient mice cloned by Oliver et al were resistant to endotoxic shock normally induced by exposure to lipopolysaccaride (LPS) [102]. This could be a result of decreased expression of genes controlled by NF-κβ [35, 97, 126, 129, 130].

The epigenetic role of PARP extends to other genomic structures besides histones. Poly (ADP-ribosyl)ation of the CCCTC-binding factor (zinc finger protein) CTCF gene, a chromatin insulator encoding protein has been shown to affect the ability of this protein to interact with over 140 target sites in mice [107, 131-133]. The role of PARP in controlling the function of the CTCF insulator in the regulation of the transcriptional states of various genes further demonstrates the role of PARP in epigenetics [134, 135].

Poly (ADP-ribose) glycohydrolase (PARG) and PARP interactions

Poly (ADP-ribose) glycohydrolase or PARG is an enzyme involved in poly (ADP-ribose) metabolism. PARG removes poly (ADP-ribose) units from proteins and thus plays an equally important role as PARP in cellular function. Though PARG is found in the cytoplasm rather than the nucleus where PARP is found [136], PARG can be transported between the nucleus and cytoplasm in order to regulate the breakdown of poly (ADP-ribose) [137]. It was found that a PARG deficiency in mice was lethal because of an accumulation of poly (ADP-ribose) [138]. Specifically, PARG maintains chromatin structure by depoly (ADP-ribosyl)ation and by acting in opposition to PARP return chromatin to its original state. PARG accomplishes this by removing poly (ADP-ribose) groups from histones and once again allowing histones to form the nucleosome structure of chromatin [137]. PARG is involved in DNA repair by regulating the amount of poly (ADP-ribose) synthesized in response to DNA damage since excessive poly (ADP-ribose) accumulation may result in cell death [101]. PARG and PARP work in opposition to each other to modify chromatin structure [117, 122]. When PARP creates transcriptionally active regions of chromatin, PARG restores chromatin to its original state. However, PARP does not always transcriptionally activate chromatin regions. For instance, in euchromatin regions, PARP is involved in chromatin de-condensation and promoting transcription while in heterochromatin regions it could repress transcription [117]. PARG deficient human A549 carcinomic basal alveolar cells showed a dramatic deficiency in their ability to repair DNA single strand breaks in the face of oxidative damage while an excess of poly (ADP-ribose) was found to be lethal for these cells [139]. Knocking out of PARG was found to be lethal in mouse embryonic cells at day 3 of gestation since PARG is the primary enzyme involved in breaking down Poly (ADP-ribose) (PAR) in cells [138].

Due to its abilities to protect against DNA damage, PARG is involved in cellular responses to oxidative stress [140]. In a recent study by Fisher et al, PARG was found to cooperate with PARP1 in responding to oxidative damage by regulating XRCC1 at DNA break sites created by oxidative damage [139]. PARG has also been shown to protect against cell damage caused by genotoxic or oxidative stress even at sub-lethal levels by regulating poly(ADP-ribosyl)ation [138].

Role of PARP in germ cell death

Apoptosis is a normal component of mammalian spermatogenesis. It is orchestrated spontaneously during the entire stages of spermatogenesis in order to produce mature spermatozoa and to eliminate any abnormal spermatozoa. In fact, a very large number of spermatozoa die and are eliminated during spermatogenesis. This may be due to the ability of the Sertoli cells to maintain only a limited number of germ cells and resulting in the elimination of excess germ cells. Apoptosis may also function to destroy cells that do not make it past certain cellular checkpoints [141] Evidence seems to point to the notion that germ cell death during mitotic and meiotic cell divisions may be needed to eliminate problems such as errors in chromosomal arrangement during meiosis or un-repaired breaks in DNA. More importantly, apoptosis may be needed to prevent genetic abnormalities from being passed onto offspring [142]. In a recent study, Codelia et al sought to determine which cell death pathway was involved in pubertal rat spermatogenesis. Using a caspase-8 inhibitor and a pan-caspase inhibitor they detected significantly less cleaved PARP and also a reduction in the number of apoptotic germ cells which suggests that germ cell apoptosis occurs via the *Fas* antigen (*Fas*)-*Fas ligand* (Fas-FasL) system and that PARP cleavage may play a key role therein [143].

Not only does PARP have a well-defined role in DNA repair, but it is also involved in apoptosis. During apoptosis, numerous DNA strand breaks can lead to PARP activation. This activation of PARP may be an attempt by the dying cell to repair the DNA damage caused by nuclease activation [129, 144, 145]. However, this attempt to repair damage proves futile as PARP is cleaved by caspase-3 into a catalytic fragment of 89 kDa and DNA binding unit of 24 kDa [61, 146]. Therefore, this cleaved version of PARP could be a biochemical marker of caspase-dependent apoptosis [147, 148].

Deregulation of germ cell death can have important implications for male fertility. Patients with contralateral testes exhibit an increased incidence of apoptosis. The presence of apoptotic markers is high in these patients especially in spermatocytes, early spermatids, late spermatids, and Sertoli cells [149]. Infertile men with spermatid and spermatocyte maturation arrest and hypospermatogenesis also showed a greater rate of apoptosis [150]. Specifically, the Fas-FasL pathway and active caspase 3 were shown to have increased activity in testes with maturation arrest and Sertoli cell-only syndrome (SCOS) [127, 151]. The greater rate of apoptosis seen with these infertility cases may be due

to elimination of germ cells with extensive DNA damage. Tesarik et al compared men with complete spermiogenesis failure to another group of azoospermic men who had incomplete failure of spermiogenesis and were able to show that apoptotic DNA damage was greater in the latter group when compared to the former. This greater extent of DNA damage seen in patients with complete spermiogenesis failure could be responsible for the low conception success rates in these cases [152]. In a more recent study, Maymon et al proposed that the presence of greater levels of poly (ADP-ribose) in human spermatocytes during maturation arrest could be correlated with the greater occurrence of DNA strand breaks during impaired spermatogenesis [153].

PARP2 has also been implicated in abnormal spermatogenesis. In a recent study by Dantzer et al, PARP2 deficient male mice were found to have hypofertility [154]. Upon examination of infertile PARP2 null mice, an increased incidence of testicular apoptosis was found specifically in the spermatocyte and spermatid layers. However, the layers containing spermatogonia and preleptotene spermatocytes largely remained normal. Chromosome segregation was abnormal during metaphase I and spindle assembly was also abnormal in these PARP2 deficient mice. Thus, the decrease in fertility seen in these PARP2-null mice could be related to defective meiosis I and spermiogenesis [81]. These results make it increasingly clear that apoptotic markers can be excellent diagnostic tools for evaluating fertility potential.

Exogenous agents applied to the testes may also activate caspase-dependent cell death pathway. For example, when the scrotal temperature was increased in rats over time, the mitochondria dependent cell death pathway in the testis was activated. The signaling cascade involved relocation of Bax, translocation of cytochrome C, activation of caspases, and the cleavage of PARP [155]. Although it is not clear what the precise role of PARP is in exogenously induced apoptosis, a study of PARP's protein targets may elucidate this role. Following exogenous stress, p53 levels increase and are considered to be a part of the mechanism that returns spermatogenesis to normal cycles following apoptosis. P53 is a downstream protein poly (ADP-ribosyl)ated by PARP [71, 75, 156], Under conditions of inflammation, PARP poly (ADP-ribosyl)ates and activates NF-κβ in human Sertoli cells [157]. Treatment with anti-inflammatory agents suppressed this cell death pathway and validated the involvement of PARP in inflammatory responses [158, 159]. It remains to be seen if this anti-inflammatory activity of PARP could also be applied to germ cells.

PARP and spermatogenesis

PARP1 has been detected in the nuclei of a variety of tissue types including the brain, heart, kidney, and testis [121]. PARP2 has been detected in the liver, kidney, spleen, adrenal gland, stomach, intestinal epithelium, thymus, brain tissue, and testis. PARP2 expression is weaker than that of PARP1 and is well distinguished [51]. PARP1 has high levels of expression in the basal regions of seminiferous tubules of developing mice,

but has almost no presence in the luminal region of the seminiferous tubules, suggesting that PARP1 is down-regulated during the haploid stage of meiosis [51].
Highest concentration of PARP1 is in rat primary spermatocytes followed by spermatids [160, 161]. In a study by Tramontano et al examining rat primary spermatocytes it was found that both PARP1 and PARP2 are present in these germ cells. However, the vast majority of poly (ADP-ribose) in these rat primary spermatocytes was produced by PARP1 suggesting possibly different roles of PARP1 and PARP2 in spermatogenesis [107]. Interestingly, PARG has also been detected in the nuclei of rat primary spermatocytes [160] suggesting the presence of a mechanism to regulate the levels of poly (ADP-ribose) in germ cells.
In a recent study using human testicular samples, it was shown that that the strongest levels of PARP1 were found in spermatogonia. Presence of poly (ADP-ribose) differed slightly with the stage of spermatogenesis. Poly (ADP-ribosyl)ation was strongest in human round and elongating spermatids as well as in a subpopulation of primary spermatocytes. In contrast, mature spermatids had no PARP expression or poly(ADP-ribosyl)ation [153]. This is in accordance with a study in rat germ cells where poly (ADP-ribose) and NAD levels progressively decreased from primary to secondary spermatocytes and to a greater extent in spermatids [74]. This decrease in PARP1 levels and activity throughout differentiating male germ cells may be correlated with the changes in chromatin structure associated with spermatogenesis. The chromatin remodeling steps of spermatogenesis include the replacement of histones by protamines [162] and a transition from a supercoiled form of DNA to a non-supercoiled form [163]. It is during these chromatin remodeling steps of spermiogenesis that DNA strand breaks can occur. In human testis, an increase in DNA strand breaks occurs in 100% of elongating spermatids [164]. These breaks were later demonstrated to be double stranded breaks caused by topoisomerase II as a result of the unique chromatin packaging steps that take place during spermatogenesis [14].
Meyer-Ficca et al (2005) demonstrated the presence of poly (ADP-ribose) (PAR) in elongated spermatids of rat [39]. They showed that during these steps when a high number of DNA breaks occur directly preceding nuclear condensation, there is correspondingly a higher amount of poly (ADP-ribose) in rat germ cells. Greater poly (ADP-ribose) formation through PARP1 and PARP2 action occurs during this phase of spermatogenesis that includes a great deal of chromatin condensation (steps 11-14 of rat spermatogenesis), poly (ADP-ribose) levels decrease only when protamines appear in the chromatin. Thus, poly (ADP-ribose) formation could be important for repairing DNA strand breaks during these crucial chromatin remodeling steps of spermatogenesis [39, 165]. Furthermore, poly (ADP-ribose) formation could also be important for histone modification because not only is there auto-modification of PARP during spermatogenesis, but much of PARP activity is targeted towards the testes-specific histone, H1t. The activation of Histone 2A (H2AX), a biological marker of DNA breaks,

and the poly (ADP-ribose)ylation of histones at break sites may act as markers of such damage [166]. Thus, the activity of PARP during the chromatin remodeling steps of spermatogenesis in terms of repairing double stranded breaks and the poly(ADP-ribosyl)ation of histones, is critical and disregulation of the chromatin remodeling steps of spermiogenesis could have serious consequences for the male gamete [164]. PARP2 knockout mouse was shown to be associated with severely compromised spermatids and delays in elongation process [167]. Heat stress has been reported to decrease PARP expression in the rat testis [50]. However heat shock protein was reported to activate the PARP and PAR formation [92].

The quest to detect PARP in ejaculated spermatozoa has met with success only recently. Taylor et al did not detect the presence of PARP1 in human ejaculated sperm samples when analyzing semen for apoptotic markers [168]. However, in a recent study by Jha et al, [169] several isoforms of PARP were detected in ejaculated spermatozoa including PARP1, PARP2, and PARP9. Immunolocalization patterns showed that PARP was found near the acrosomal regions in sperm heads. Furthermore, a direct correlation was seen between sperm maturity and the presence of PARP, i.e., an increased presence of PARP1, PARP2, and PARP9 was seen in mature sperm when compared to immature sperm. Just as importantly, the level of PARP1, PARP2 and PARP9 was more in ejaculated sperm from fertile men when compared to infertile men indicating a possible relationship between PARP and male infertility. PARP activity was then modulated to determine its role in the response to oxidative and chemical damage in sperm. In the presence of a PARP inhibitor, 3-aminobenzamide, chemical and oxidative stress-induced apoptosis increased by nearly two-fold. This novel finding suggests that PARP could play an important role in protecting spermatozoa subjected to oxidative and chemical damage [169].

PARP and sperm DNA

Recently the importance of DNA damage to sperm of infertile men has gained attention. It has been shown that that a higher index of DNA damage can possibly lead to lower semen quality. In a study involving 322 couples, when DNA fragmentation exceeded an index of 15%, there was increased incidence of non-transfer and miscarriages after performing intracytoplasmic sperm injection (ICSI) [170]. Studies such as these warrant the investigation of factors that are responsible for maintaining genomic integrity especially in ejaculated spermatozoa. DNA damage repair through PARP activity has been demonstrated in rat germ cells. Atorino et al showed that after extensive DNA damage in rat spermatids and spermatocytes caused by radiation and ROS damage *in vivo*, a PARP inhibitor caused a delay in DNA damage repair [40]. They were able to demonstrate up to a 3-fold increase in PARP activation as cells recovered from these damaging agents [60, 125, 126]. The main difference between spermatids and primary spermatocytes was that only spermatids showed detectable poly (ADP-ribose)

production after genotoxic stress. Atorino et al hypothesized that primary spermatocytes did not show the same degree of response possibly due to the different chromatin states of these two germ cells. Thus, PARP activity in response to genotoxic stress may be important for preventing mutations from accumulating and being passed on to offspring [40].

Though the role of PARP in repairing DNA damage in ejaculated spermatozoa has yet to be thoroughly investigated, it has been found that DNA damage caused by sperm cryopreservation can be repaired through PARP activity. In a study by Kopeika et al, cryopreserved sperm from loach (fresh water fish related to crab) were used to fertilize eggs and the embryos were exposed to a PARP inhibitor [171]. It was found that survival was significantly decreased in embryos exposed to PARP inhibitors when compared to control. This study suggests a possible role for PARP in repairing paternal DNA damage and it also showed that it was possible for the oocyte to repair this damage even in the face of PARP inhibition [171].

Cryopreservation is not the only threat to genomic integrity. It is still controversial whether malignancy itself is the cause of chromatin damage in mature spermatozoa. In a study analyzing 75 men with various types of testicular and non-testicular cancers, the degree of DNA fragmentation did not differ between types of cancer. But surprisingly, the levels of DNA fragmentation in cancer patients were similar to the levels found in infertile men [172]. Another study found that patients with testicular cancer and Hodgkin's Lymphoma were normospermic, but had increased levels of DNA damage along with decreased chromatin compaction [173]. However, a third study in the same year hypothesized that malignancy alone was not responsible for increased DNA damage seen in sperm. This study showed that thawed sperm samples from cancer patients had similar levels of DNA fragmentation to that of sperm with low freezability collected from healthy donors. Thus, this study attributed the increased sperm DNA damage seen in the decreased freezability of these semen specimens and not to malignancy [174].

Nonetheless, cancer patients are advised to freeze sperm prior to therapy as an option to preserve their fertility potential. Patients undergoing radiotherapy have shown a temporary increase in the sperm with DNA strand breaks and a decrease in fertility despite having normal sperm concentrations (normozoospermic) [42]. Chemotherapy does not produce the same deleterious effects as radiotherapy in its initial phase. Decrease in sperm DNA fragmentation is generally seen after 3 or more cycles of chemotherapy [175]. Chemotherapy (especially alkylating agents) and radiotherapy, even in low doses, can damage the seminiferous epithelium and impair spermatogenesis in both children and adults [176].

Apart from treatment by either chemo- or radiotherapy, malignancy itself causes a significant threat to sperm DNA damage. This could mean that cryopreservation of sperm prior to therapy may not be sufficient to preserve fertility [175]. Instead, therapeutic

options such as PARP activity modifications could be used in order to retain genomic integrity under threats of malignancy and radiotherapy, so that we may get rid of the low quality spermatozoa that have DNA damage [177, 178].

Sperm DNA damage repair defects

DNA polymerase activity in non-replicating cells is associated with DNA repair. Consequently, increase in apoptotic markers seen in the semen of infertile men may also be an indicator of increase DNA repair activity [15]. Evidence from the literature indicates that DNA repair systems may play a role during spermiogenesis. Elements of base excision repair (BER) have been identified in elongated spermatids [179]. Mismatch repair (MMR) involving the mutS homolog 2 (MSH2) proteins has a role in spermiogenesis. Interestingly, in a mouse model for Huntington disease, deletion of MSH2 in Huntington disease abolished trinucleotide cytosine-adenine-guanisine (CAG) repeat expansion between round spermatids and spermatozoa. CAG, is a DNA mutation responsible for causing any type of disorder categorized as a trinucleotide repeat disorder. Presence of CAG repeat expansion may explain the earlier onset of the disease and the severity of the symptoms through successive generations of an affected family due to the expansion of these repeats. Deletion of MSH2 suggests an active role for MSH2 during extensive DNA repair [180-183]. Quite interestingly, caffeine may lead to inactivation of H2AX and non-homologous end-joining (NHEJ) DNA repair [184]. Impairment in DNA repair during spermiogenesis may result in persistent double stranded breaks in mature spermatozoa. Further investigation may provide important clues regarding the consequences of the endogenous DNA strand breaks and repair in spermatids and mature spermatozoa [185].

Down regulation of DNA repair genes such as Ogg1 (involved in base excision repair), Rad54 (involved in double-strand break repair) and Xpg (involved in nucleotide excision repair) has been reported using global genome expression by DNA microarray following exposure to heat stress at 43^{o}C [30]. Heat stress induced by cryptorchidism appears to result in decreased expression of DNA polymerase B and DNA ligase III both of which are involved in the final stages of DNA repair [186, 187].

PARP - a new marker in ejaculated spermatozoa

The role of PARP in DNA repair (Figure 2) and its presence during stages of spermatogenesis suggest that it is involved in maintaining genomic integrity in ejaculated sperm. However, the presence of PARP has only recently been shown in ejaculated sperm samples. Furthermore, Jha et al have suggested a correlation with the presence of PARP and male fertility [169]. This study suggested that the decreased presence of PARP in the sperm of infertile men could be the cause of increased DNA damage seen in poor quality semen samples. DNA damage repair in germ cells as mediated by PARP is therefore a hitherto unexplored confounder of male fertility. Further studies are

needed to fully explore the role of PARP in DNA repair especially in reproductive medicine [50-52, 55, 188].

The existing methods of detecting infertility from semen samples are quantitative methods involving the count and observation of sperm and are dubious due to the constantly changing 'normal' seminal values. A qualitative method would thus serve as a more reliable method of detecting infertility [125, 162]. In view of this, the detection of apoptotic markers such as caspase and phosphatidylserine have been successfully correlated with a variety of infertility conditions; however, the use of PARP as an apoptotic marker has not been fully explored.

Taylor et al found greater caspase activity in low motility sperm samples from infertile men when compared to those with high motility [168]. The active caspase enzymes have been localized in the human spermatozoa predominantly in the post acrosomal region (caspase 8, 1 and 3) [189-191] or in the mid-piece [192]. A significant positive correlation between **in**-situ active caspase-3 **in** the sperm midpiece and DNA fragmentation was observed **in** the low motility fractions **of** patients, suggest**in**g that caspase-dependent apoptotic mechanisms could orig**in**ate **in** the cytoplasmic droplet or with**in** mitochondria and function **in** the nucleus [193]. These data suggest that **in** some ejaculated sperm populations, **caspases** are present and may function to **in**crease PS **translocation** and DNA fragmentation. Furthermore fluorescence staining of active caspases localized the enzymes mainly to the postacrosomal region in sperm from donors. This pattern differed slightly from that of patients, in whom additional cytoplasmic residues were found to be highly positive [194].

In an interesting study published by Falerio and Lazebnik in 2000, question as to how caspase 3 which is usually cytoplasmic gains access to its nuclear targets was examined [195]. These investigators suggested that caspase-3 was actively transported to the nucleus through the nuclear pores. They found that caspase-9, which is activated earlier than caspase-3, directly or indirectly inactivated nuclear transport and increased the diffusion limit of the nuclear pores. This increase allowed caspase-3 and other molecules that could not pass through the nuclear pores in living cells to enter or leave the nucleus during apoptosis by diffusion. Hence they suggested that caspase-9 contributes to cell disassembly by disrupting the nuclear cytoplasmic barrier [195].

Unlike somatic cells, early studies were not able to detect PARP1 or AIF (which is activated by PARP) in ejaculated sperm [106, 168, 196, 197]. Similarly, the presence of cleaved PARP could not be detected in ejaculated sperm [198]; although PARP activity was demonstrated in the testis [199]. We studied the localization of the PARP in mature and immature spermatozoa in fertile and infertile men [169]. Mahfouz et al were the first to demonstrate the presence of cleaved PARP in ejaculated spermatozoa. When these sperm samples were exposed to PARP inhibitors after chemical and oxidative stress, there was a decreased incidence of apoptosis [200]. These authors did not study PARP

and caspase 3 co-localization and they proposed that PARP cleavage may occur by activated caspase 3 located in the post acrosomal region. It would be worthwhile to replicate these findings in other mammals and investigate the use of cleaved PARP as a diagnostic tool to predict/ detect male infertility.

PARP and oxidative stress

Oxidative stress (OS) occurs when there is an increase in the levels of reactive oxygen species (ROS) and/ or a decrease in the activity of the antioxidant enzymes that scavenge these harmful free radicals [201, 202]. Such conditions of oxidative stress arise when germ cells are faced with biological (such as lipopolysaccharide, LPS) or chemical stressors (e.g. environmental toxicants, endocrine disruptors etc.) [20, 31, 203-205]. The extent of oxidative stress induced depends on the dose and duration of exposure to the stressor [205]. Oxidative stress can cause modification of proteins associated with developing spermatozoa and cause the premature release of sperm from seminiferous tubules [206]. Even a transient state of oxidative stress, spanning a few hours has been shown to bring about protein changes and stimulate a caspase-3-mediated cell death pathway and apoptosis [43, 207]. In the reproductive milieu, oxidative stress is also linked to inflammation particularly since inflammatory cytokines dramatically arrest spermatogenesis and may lead to infertility [208]. However, the most important effect is the ability of oxidative stress to cause DNA damage. PARP responds to all three of these changes that can occur in the cell as a result of oxidative damage. However, there is a great deal of variability in PARP activation as a result of this type of stress depending on the metabolic stage of the cell or its microenvironment [209-211]. As a result of its interaction with NF-κβ, PARP has an important role in the inflammation process [78, 102]. In response to DNA damage caused by oxidative species, PARP1 recruits the DNA repair protein XRCC1 to the sites of the damage [212]. In addition to causing damage to DNA, oxidative species can harm histones wherein PARP activates the 20S proteosome involved in breaking down oxidatively damaged histones. Also, in response to oxidative stress caused by exposure of histones to hydrogen peroxide, a complex is formed by the binding of PARP, poly (ADP-ribose), and the nuclear proteosome [133, 213]. This complex formation could be important because a condensed chromatin structure may protect DNA from strand breaks induced by hydroxyl radicals [197, 214].

In conditions of oxidative stress-induced necrosis in Bax-/- Bak -/- (a proapoptotic protein that regulates the intrinsic apoptotic pathway) mouse embryonic fibroblasts, there is an activation of PARP1. PARP1-catalysed poly (ADP-ribosyl)ation causes a depletion of ATP, which promotes the autophagy of these necrotic embryonic cells [215]. PARP1 has been implicated in the repair of DNA damaged by estradiol in human estrogen-receptor-negative (ER -/-) breast cancer cells. Treatment of these breast cancer cells with 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), which is itself an estrogenic toxicant, altered the expression of enzymes responsible for the bio-activation of

estrogen leading to DNA damage, PARP1 activation and DNA repair. Thus, the apoptosis of human ER -/ breast cancer cells was prevented by TCDD-induced activation of PARP1 and aided the survival of these cancer cells [216]. Interestingly, administration of TCDD caused the expression of cDNA encoding a 75 kDa protein that had sequence similarity with PARP. This protein, renamed as TCDD-inducible PARP (TiPARP) possessed catalytic activity similar to PARP [217]. However, the role of TiPARP on chromosome stability, DNA repair and apoptosis are yet to be elucidated.

PARP and ageing

The ageing process also takes its toll on DNA, which can in turn, affect the fertility potential of a male. As discussed earlier, PARP is also involved in ageing through its role in immune responses, telomere maintenance, DNA repair, spindle assembly, and cell death [218]. In a recent study, El-Domyati et al collected sperm samples from fertile men of various age groups and quantified the differences in PARP1 presence and PARP activity [199]. The expression of PARP1 and its DNA repair partner, XRCC1 were higher in spermatocytes of older men while the Sertoli cells of these men showed higher poly (ADP- ribose) levels. Apoptosis was increased in older men who showed more active caspase-3 and cleaved PARP1 in the spermatogonia and spermatocytes. This general increase in PARP1 and DNA repair enzymes could be associated with this declining DNA integrity as a result of age [199].

Chromosomal stability is vital for the survival of an organism and chromosomal instability increases with the age of an organism and is considered a symptom of ageing. PARP is essential for chromosomal stability through its role in DNA repair. PARP activity may influence ageing by maintaining genomic stability through DNA repair, telomere maintenance, spindle stability, and cell death [210, 218-220]. Under physiological conditions, both PARP1 and PARP2 affect telomere functioning by binding to telomere repeat binding factor II (TRFII) and affecting its ability to bind to telomere regions [221-223]. PARP1 is also found at telomere regions of DNA that is damaged by genotoxic agents and it may play a role in preventing damage to genomic stability [222]. PARP1 deficient mice showed greater incidence of chromosomal aberrations, polyploidy and telomere shortening. When PARP was reintroduced in the form of cDNA, they were then able to restore chromosomal integrity. [224, 225]

Biological role of PARP in male fertility

PARP plays a crucial role in maintaining genomic integrity in a variety of cell types and perhaps nowhere is this genomic integrity more important than in germ cells. Cases of male infertility are associated with abnormal sperm chromatin and DNA structure. The problems that arise in genomic integrity of sperm come from a variety of sources including spermatogenesis defects, abortive apoptosis, problems with spermatid maturation, and oxidative stress [22, 226]. Problems in spermatogenesis could include

double strand breaks that are not resolved after crossing over during meiosis I [22]. Although there has not been any clear association between apoptotic markers and DNA fragmentation in mature male gametes, it is possible that incomplete apoptosis could be a cause of such DNA fragmentation [22, 227]. Maturation of spermatids involves chromatin remodeling steps that involve necessary DNA strand breaks and thus could be a source of unresolved breaks. Lastly, reactive oxidative species (ROS) have been extensively investigated in male infertility, and in light of the activation of PARP-induced apoptosis pathways in oxidative stress conditions, may provide an explanation for the role of PARP in male fertility.

Thus, at the time of writing this review, the role of PARP in male fertility is not as well defined as its role in other cellular processes. However, there is enough evidence such as detection of PARP in the testis, during spermatogenesis, and in ejaculated spermatozoa to suggest that such a role exists [169, 200]. Furthermore, the role of PARP as an important DNA repair enzyme could empower it with maintaining the genomic integrity of sperm. Similarly, the role of PARP in cell death pathways may have important implications for its role in the elimination of abnormal spermatozoa during the processes of spermatogenesis [228].

Potential therapeutic applications of PARP

The key role of PARP in cell death has made it an attractive candidate in cancer therapies [157]. It is based on the simple idea that inhibiting DNA repair in malignant cells exposed to chemotherapy will kill off these cancerous cells due to the large amounts of DNA damage that will accumulate if PARP is inactivated [229]. Non-malignant cells will not be susceptible to cell death at these low doses of chemotherapy. Thus PARP inhibitors could provide a means to sensitize cancerous cells to chemotherapy and be developed as an adjuvant to chemotherapy [230]. An alternate strategy being explored is that of inactivating PARP through cleavage to attain the same end result, i.e., accumulation of damaged DNA in cancerous cells causing them to die faster [228, 231].

PARP modulation may not only prove useful in cancer therapies, but also in dangerous inflammatory processes. The role of PARP in inflammation especially through the recruitment of NF-κβ and through its role in responding to oxidative stress produced by the inflammatory processes make it a powerful target for anti-inflammatory therapy [157]. The use of PARP inhibitors as therapeutics in conditions such as cerebral ischemia and other inflammation-induced conditions may be explored [159, 220]. It still remains to be seen whether PARP can provide a therapy for male infertility. PARP inhibition may protect against chemically induced injury of ejaculated spermatozoa in vitro, but was not effective against damage induced by oxidative stress [200]. It is also possible that PARP inhibition may have a potential role in testicular cancer as well as cancer that may have spread to the testes [109]. Inflammatory processes as result of infections could also be another area to explore in terms of PARP and male fertility.

Conclusions

PARP homologues have diverse role(s) in spermatogenesis and in ejaculated sperm. PARP expression is associated with sperm maturity in proven fertile men. PARP inhibition may be used as an *in vitro* treatment in certain conditions such as oxidative stress and/ or chemically induced death of spermatozoa with damaged DNA. Therefore these spermatozoa may not have the ability to fertilize or produce healthy embryo as the oocyte repair system may be inadequate to correct such high DNA damage. PARP modulation using kinase activators or inhibitors may have a future beneficial role in infertile patients exhibiting sperm DNA damage. This could pave the way for future studies to elucidate the role of PARP in other conditions resulting in sperm DNA damage. Cleaved PARP, which is activated during apoptosis, could serve as an apoptotic marker for differentiating healthy spermatozoa from apoptotic ones. In addition, the anti-tumor properties of PARP could provide new strategies to preserve fertility in cancer patients even after genotoxic stresses like radiation. The possibility of using DNA damaged sperm in ART especially in ICSI needs careful evaluation. PARP may hold the key to a better understanding of these repair mechanisms inherent in spermatozoa and the importance of such mechanisms in producing healthy pregnancies.

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Abbreviations

AIF	Apoptosis inducing factor
AMD	Auto modification domain
ADPR	ADP ribose
AP	Apurinic-apyrimidinic endonuclease
ART	Assisted reproductive technologies
BER	Base excision repair
BRCA-1 CT	Breast cancer-1, human oncogene, C-Terminus
Bub3	Budding uninhibited by benzimidazoles 3
CAG	Tri-nucleotide (Cytosine- Adenine- Guanine)
CD	Catalytic domain
CTCF	CCCTC-binding factor (zinc finger protein)
DBD	DNA binding domain
DBD	DNA binding domain
ERK	Extracellular signal-regulated kinases
Fas-FasL	*Fas* antigen (*Fas*)/*Fas ligand* (*FasL*)
H2AX	One of several genes coding for histone H2A
ICSI	Intracytoplasmic sperm injection
LPS	Lipopolysaccaride
MALDI-TOF-TOF	Matrix-assisted laser desorption/ionization time-of-flight/ time-of-flight
MSH2	MutS homolog 2
NAD	Nicotinamide adenine dinucleotide
NBS1	Nijmegen Breakage Syndrome 1
NF-κβ	Nuclear factor kappa β
NHEJ	Non-homologous end-joining
NLS	Nuclear localization signal
PAR	Poly (ADP-ribose)
PARG	Poly (ADP-ribose) glycohydrolase
PARP	Poly (ADP-ribose) polymerase
Rad54	A gene linked to chromosome 1p32, encodes for a protein known to be involved in the homologous recombination and repair of DNA.
ROS	Reactive oxygen species
SCOS	Sertoli cell only syndrome
SIRT1	Silent information regulator gene in human affect the metabolism and inflammation
TBP	TATA-binding protein
TCDD	2, 3, 7, 8-tetrachlorodibenzo-p-dioxin
TRFI-I	Telomere repeat binding factor II, I
WWE domains	Domain associated with protein-protein interaction
XRCC1:	X-ray repair complementing defective repair in Chinese hamster cells 1
YY1	YinYang1

Chapter 3

Evaluation of poly (ADP-ribose) polymerase cleavage (cPARP) in ejaculated human sperm after induction of apoptosis

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Abstract

Objective: To examine the presence of cleaved poly(ADP-ribose) polymerase(s) (cPARP) in ejaculated spermatozoa and determine cPARP levels following exposure to chemical or oxidative stress.
Design: Prospective pilot study.
Setting: Tertiary care academic hospital.
Patient(s): Eight healthy men.
Intervention(s): Semen specimens were collected, prepared with double-density-gradient centrifugation, and divided into control, hydrogen peroxide (H_2O_2), H_2O_2 + 3-aminobenzamide (3-ABA), staurosporine (STS), and STS + 3-ABA treated groups.
Main Outcome Measure(s): Cleaved PARP and apoptosis markers by flow cytometry.
Result(s): Cleaved PARP was detected in both neat and mature fractions. The cPARP levels were similar in both mature and immature spermatozoa. In combined mature and immature fractions, a higher percentage of late apoptotic sperm was seen in STS + 3-ABA versus STS. Higher levels of late apoptotic spermatozoa were seen in immature versus mature fractions within STS and STS + 3-ABA groups. Lower levels of cPARP were seen in immature versus mature fractions in H_2O_2 and H_2O_2 + 3-ABA treated groups. Cleaved PARP was related to levels of activated caspase-3.
Conclusion(s): Cleaved PARP is present in ejaculated human spermatozoa. Poly (ADP-ribose) polymerase inhibitors may play a different role in chemical versus oxidative stress-induced sperm damage.

Key words: Apoptosis; flow cytometry; PARP cleavage; sperm fraction; TUNEL

Introduction

Male factor infertility is solely responsible for about 20% of infertility cases and is contributory in another 30 to 40% [1, 2]. The possible etiologies of male infertility include idiopathic causes, varicocele, extensive iatrogenic interventions, and erectile dysfunction, as well as defective spermatozoa [3-7]. One possible cause of male infertility that has been studied intensely in the last decade is the integrity of DNA in the nucleus of mature ejaculated spermatozoa [8, 9]. There are many possible causes of sperm DNA damage, including abortive apoptosis, oxidative stress associated with infections, chemical exposure, and defects of spermiogenesis associated with the retention of excess residual cytoplasm [10-16]. The focus on the genomic integrity of the male gamete has been intensified by the growing concern about transmission of genetic diseases through intracytoplasmic sperm injection (ICSI) [17].

Apoptosis is defined as programmed cell death, which is an energy- and protein-dependent process that depends upon the cellular ATP level [18]. Human spermatozoa have the ability to undergo apoptosis or apoptosis-like conditions [19-27]. Absence of Fas protein from the spermatozoa or dysfunction has been reported [28, 29], and another study reported presence of Fas ligand on the mature ejaculated spermatozoa [30-32]. Sperm DNA damage can occur at the time of, or be the result of, DNA packaging during spermiogenesis stages through abortive apoptosis [33-37].

Poly(ADP-ribosyl)ation is a DNA damage-dependent process that is affected by cell energy level. Poly(ADP-ribose) (PAR) level is controlled by the opposing actions of poly(ADP-ribose) polymerase (PARP) and poly(ADP-ribose) glycohydrolase (PARG), a major enzyme responsible for the catabolism of PAR. PAR metabolism is critical in a wide range of biological structures and processes. These include DNA repair and maintenance of genomic stability, transcriptional regulation, centromere and centrosomal function, mitotic spindle formation, telomere dynamics, trafficking of endosomal vesicles, apoptosis, and necrosis [38-41]. In humans, the PARP family is comprised of as many as 18 distinct proteins; PARP-1 is the most expressed member [39]. PARP-1 plays a primary role in the process of poly-ADP-ribosylation. It has been implicated in genome maintenance, carcinogenesis, aging, immunity, inflammatory conditions, and neurological function [40, 42].

Poly(ADP-ribose) polymerase 1 has a well-characterized role in DNA damage detection and repair. Protein ribosylation by PARP-1 is an immediate response to DNA damage induced by oxidants, alkylation, or ionizing radiation [40, 43]. In contrast to PARP-1's role as a survival factor in limited DNA damage, PARP-1 promotes cell death under conditions of extensive DNA damage [38]. Therefore, chemical inhibition or genetic ablation of PARP-1 can provide protection against cell death, including ischemia-perfusion injury, cardiac infarction, neural cell damage, or aging [44-46].

Poly(ADP-ribose) polymerase has been documented consistently as playing a functional

role in testicular germ cells [47-49]. Germ cells require PARP for repair of DNA damage [50]. Maymoun et al. detected PAR expression in germline cells [51]. Its subcellular localization in meiotic and post-meiotic prophases demonstrated chromatin modifications occurring during spermatogenesis stages demonstrated chromatin modifications occurring during spermatogenesis. Although spermatids do not enter a prophase, Maymon et al. concluded that PARP expression in germline cells and its subcellular localization in meiotic and postmeiotic prophases demonstrate chromatin modifications occurring during spermatogenesis. Their results showed that PARP was localized in germ cell nuclei in full spermatogenesis (in round and elongated spermatid and primary spermatocytes). This established a key role for PAR in germ cell differentiation, presumably to safeguard DNA integrity [49, 51].

Besides the established central role of PARP-1 and PARP-2 in the maintenance of genomic integrity, accumulating evidence indicates that PAR may modulate epigenetic modifications under physiological conditions. Poly(ADP-ribose) polymerase 2 exerts essential functions during meiosis I and haploid gamete differentiation [52]. However, a similar role for PARP in human ejaculated spermatozoa is controversial. Whether PARP can repair DNA damage in mature human spermatozoa remains largely unclear. Oocyte repair capacity will help in the presence of sperm DNA damage. However, recently PARP was shown to have a role in full spermatogenesis, and we have reported its presence in different homologues. Poly(ADP-ribose) polymerase may protect the sperm DNA during the maturation/transportation processes. In addition, it may play a role in sperm chromatin remodulation.

Conflicting reports exist regarding the presence of whole or cleaved PARP [25, 53]. Recently we reported the presence of PARP homologues in ejaculated human spermatozoa and identified these as PARP-1 (~75 kDa), PARP-9 (~63 kDa), and PARP-2 (~60 kDa) [54]. We demonstrated a positive correlation between the amount of PARP protein and sperm maturity, suggesting a role for PARP proteins in sperm DNA damage/repair.

The goal of our study was to validate the presence of cPARP in ejaculated human spermatozoa and determine whether the cPARP level can be modulated by chemical or oxidative stress cell injury in the presence or absence of PARP inhibitor.

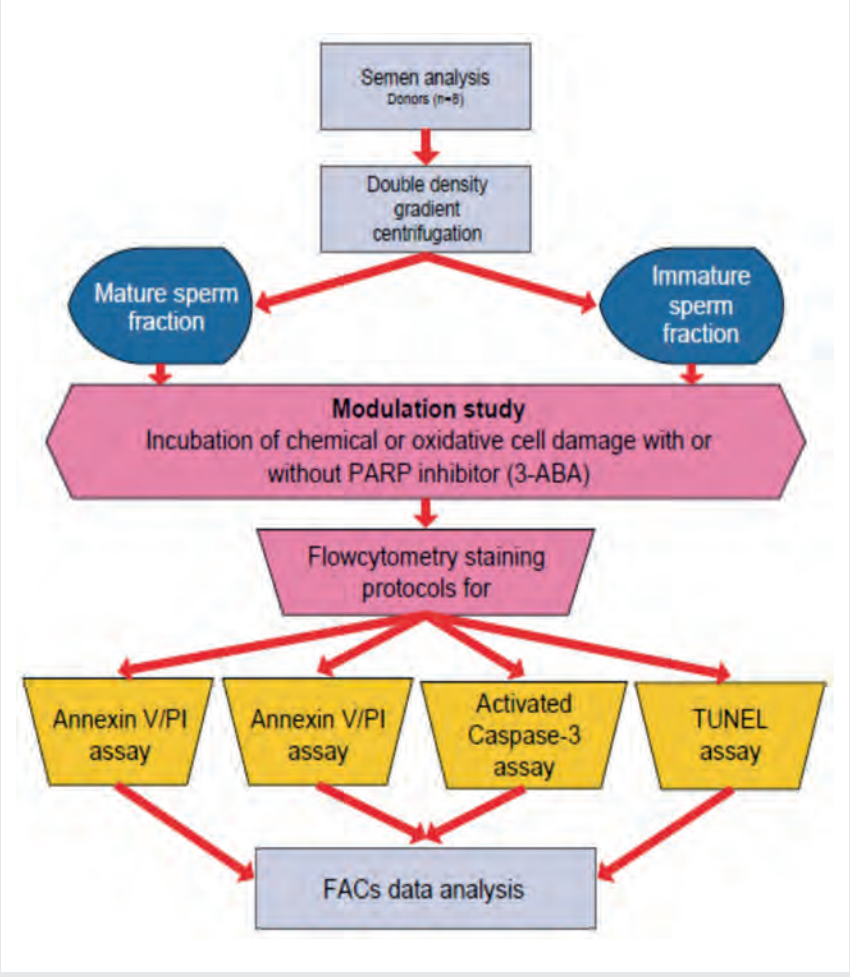
Materials and Methods

Sample Collection

A pilot study was conducted following Institutional Review Board approval. Following 48 – 72 h of sexual abstinence, semen samples were collected from eight healthy donors, defined as a group of healthy male volunteers with sperm parameters in the normal range (normozoospermic) according to World Health Organization (WHO)

guidelines [55]. These included men with proven and unproven fertility. Sample collection was done at the Cleveland Clinic Andrology Laboratory by masturbation into sterile containers. Flowchart of our experimental design of this study is shown in Figure 1.

Figure 1 Flowchart of various steps involved in processing of semen sample after collection, modulation and analysis for various parameters. 3-ABA, 3-aminobenzamide; cPARP, cleaved poly (ADP-ribose) polymerase; FACS, fluorescence activated cell sorter; PI, propodium iodide; TUNEL, terminal deoxynucleotidyl transferase mediated fluorescein-dUTP nick-end labeling.



Standard Semen Analysis

After liquefaction, semen specimens were evaluated for volume, sperm concentration, total cell count, motility, and morphology. A 5µl aliquot of the sample was loaded on a Microcell slide chamber (Conception Technologies, San Diego, CA) for manual evaluation of concentration and motility.

Separation of Mature and Immature Sperm Population

Liquefied semen (1-2 ml) was loaded onto a 40% and 80% discontinuous PureCeption® density gradient (SAGE Assisted Reproduction Products, Bedminster, NJ) and centrifuged at 1600 rpm (400g) for 20 min at room temperature. The resulting 80% pellet, representing the mature fraction, and 40% cell layer, representing the immature fraction, were separately suspended in human tubal fluid (HTF) media (Irvine Scientific, Santa Ana, CA) and centrifuged at 300*g* for an additional 7 min to remove the remaining media.

The interphase between the 40 to 80% gradient comprising the immature sperm was carefully aspirated into a tube, washed and re-suspended in sperm wash medium. This fraction consisted largely of immature, morphologically abnormal sperm with poor motility. Similarly, the pellet at the bottom of the 80% gradient was carefully aspirated into another tube, washed and re-suspended in sperm wash medium. This fraction consisted of morphologically normal, highly motile, mature sperm. Both the mature and immature sperm fractions were washed with Quinn's sperm wash media (SAGE In Vitro Fertilization Inc., San Clemente, CA), and final washing was done with phosphate buffer saline (PBS, pH 7.4).

Induction and Modulation of Apoptotic and Oxidative Sperm DNA Damage

In vitro sperm DNA damage was induced using: 1) staurosporine (STS, 10µM ; Sigma-Aldrich, St Louis, MO) incubation at 37°C for 4 h [56]; and 2) hydrogen peroxide (H_2O_2, 100µM; Sigma-Aldrich) incubation at 37°C for 1 h [57]. PARP inhibition was achieved using 3-aminobenzamide (3-ABA, 1mM; Sigma-Aldrich) [58].

Assessment of Cleaved Poly(ADP-Ribose) Polymerase (c-PARP)

The presence of c-PARP was detected using an FITC-conjugated anti-PARP cleavage site-specific antibody (CSSA) kit (ApoTarget, Anti-PARP CSSA FITC apoptosis detection kit, BioSource International Inc., Camarillo, CA) as described earlier [59]. Briefly, sperm pellets were washed twice with PBS by centrifugation at X300g, and the intracellular staining protocol was followed according to the manufacturer's instructions. Sperm pellets were fixed in IC Fix™ buffer for 20 min at 4°C at concentration of 1mL IC Fix™ buffer per 1 x 10^6 sperms. Tubes were spun at X300*g* for 5 min; the supernatant was discarded, and the pellets were washed twice in PBS. Cells were re-suspended in IC Perm™ buffer to yield a density of 1 x 10^6/ 50µl. 10µl of FITC conjugated anti-PARP

CSSA was incubated with the treated and untreated sperm cell suspensions at room temperature for 30 min. Samples were washed twice in 2ml IC Perm™ buffer and once in PBS and re-suspended in 0.5ml PBS for FACS analysis.

Terminal Deoxynucleotidyl Transferase-Mediated Fluorescein-dUTP Nick End Labeling Assay

Sperm DNA fragmentation was evaluated using the terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL) assay kit (Apo-Direct™, BD Biosciences Pharmingen, San Diego, CA) as described earlier [60-63]. Briefly, 1 x 10^6 spermatozoa were washed in PBS, re-suspended in 1% paraformaldehyde and placed on ice for 30-60 min. Subesequently, spermatozoa were washed again and re-suspended in 70% ice-cold ethanol.

Following a second wash in PBS to remove the ethanol, sperm pellet samples as well as the positive and negative controls provided with the assay kit were re-suspended in 50µl of the staining solution for 60 min at 37°C. The staining solution contained terminal deoxytransferase (TdT) enzyme, TdT reaction buffer, fluorescein-tagged deoxyuridine triphosphate nucleotides (FITC-dUTP) and distilled water. All cells were further washed in rinse buffer, re-suspended in 0.5ml of propidium iodide/RNase solution and incubated for 30 min in the dark at room temperature followed by flow cytometric analysis.

Annexin V/ Propidium Iodide Assay

To perform this assay, the Annexin-V FITC Apoptosis Detection Kit was used (BD Biosciences Pharmingen). A 100µl aliquot of neat, mature, and immature sperm fractions was re-suspended in 400µl cold reaction buffer (HEPES; N2-hydroxyethyl piperazine-N'2-ethanesulfonic acid) containing 2.5mM $CaCl_2$. Sperm cells were labeled with 10µl each of annexin-V/FITC solution and propidium iodide (PI) for detecting apoptotic and necrotic sperm. Samples were incubated for 15 min at room temperature in the dark. Cells were washed with 1ml PBS, centrifuged and re-suspended in 300µl of reaction buffer. Flow cytometric analysis was done to quantitatively determine the percentage of early and late apoptotic, necrotic, and viable cells. Early apoptotic sperm were defined as sperm that were positive for only annexin V (annexin V^+), while late apoptotic sperm were those that were positive for both annexin V and PI (annexin V^+/ PI^+) [64-66].

Active Caspase-3 Staining

Assessment of active caspase-3 activity was performed using a phytoerythrin-conjugated monoclonal active caspase-3 antibody apoptosis kit (BD Biosciences Pharmingen) as established earlier [67]. Briefly, sperm pellets were washed twice with cold PBS, re-suspended in 0.5 ml Cytofix/Cytoperm™ solution/ x 10^6 sperm and

incubated with the cytofix for 20 min on ice. Centrifugation was done at 300*g*; supernatant was aspirated and discarded. Sperm pellets were washed twice with 0.5 ml Perm/Wash buffer™/ x10^6 sperm. For each test tube, 100 µl Perm/Wash buffer and 20 µl antibody were added and incubated for 30 min at room temperature. Washing was done in 1.0 ml Perm/Wash buffer followed by re-suspension in 0.5 ml of the buffer solution. All samples were analyzed by flow cytometry.

Flow Cytometry (FCM) Analysis

All fluorescence signals of labeled spermatozoa were analyzed by the flow cytometer FACScan (BD Biosciences). Approximately 10,000 spermatozoa were examined for each assay at a flow rate of <100 cells/sec. The excitation wavelength was 488 nm supplied by an argon laser at 15 mW. Green fluorescence (480–530 nm) was measured in the FL-1 channel and red fluorescence (580–630 nm) in the FL-2 channel. Gating was done to exclude debris and aggregates using 90° and forward-angle light scatter. The percentage of positive cells and the mean fluorescence were calculated on a 1023-channel scale using the flow cytometer software FlowJo version 6.4.2 (Tree Star, Inc., Ashland, OR).

Statistical Analysis:

Data were represented as mean ± SD or median (25^{th} -75^{th} percentiles). We used the Wilcoxon signed-rank test for comparisons between groups and the Spearman non-parametric test for correlations. All statistical analysis was done by using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). A P value <0.05 was considered statistically significant.

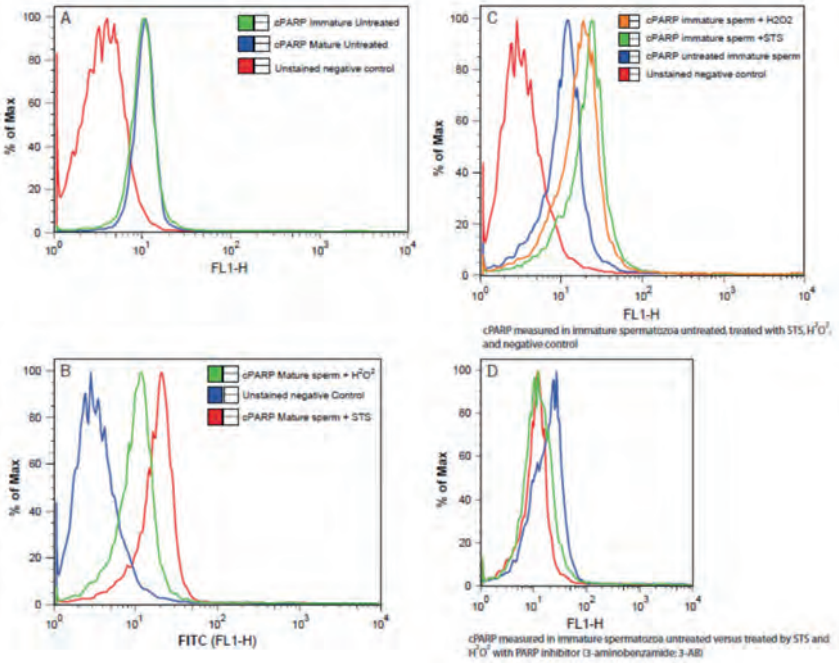
Results

Ejaculated sperm had a semen volume of 2.85 ± 1.17 ml; concentration 80.2 ± 50.5 x 10^6/ ml; median (25^{th} and 75^{th} percentiles) 65.9 (48.7, 136.1), respectively; and sperm motility (%) 64.2 ± 9.15.

Apoptotic Markers in Mature and Immature Sperm without PARP Modulation

Detectable levels of cPARP were seen in both mature and immature spermatozoa using the intracellular staining technique followed by detection with flow cytometry (Figure 2). The percentage of sperm that were positive for cPARP was comparable in both mature and immature sperm (Figures 3).

Figure 2 Overlay histograms for cPARP %+ve sperm: **(A)** Mature vs. immature with the negative control; **(B)** Oxidative vs. chemical stress-induced damage with unstained negative control in mature spermatozoa; **(C)** Oxidative vs. chemical stress-induced cell damage with unstained negative control in immature spermatozoa; **(D)** effect of PARP inhibition in oxidative or chemical stress-induced cell damage on PARP cleavage.



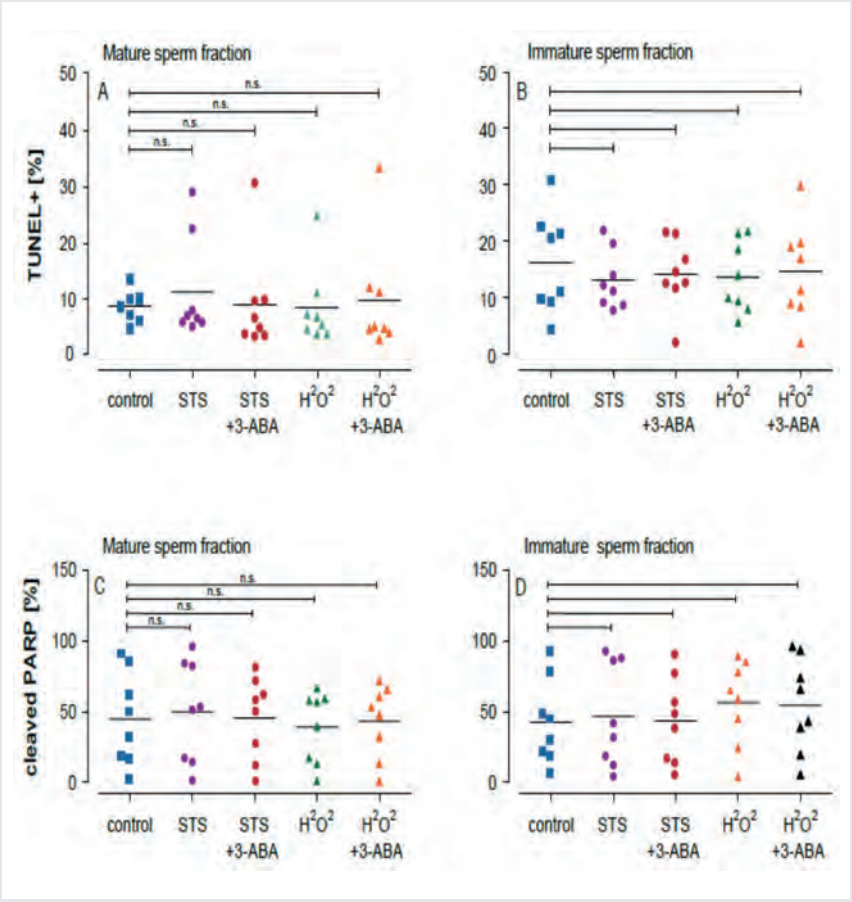
PARP Modulation Experiment in the Presence and Absence of Inducer/Inhibitor

Mature and immature sperm were exposed to PARP modulation by chemical injury (STS) or an oxidative stress inducer (H_2O_2) in the presence or absence of the PARP inhibitor 3-ABA. Results are shown in Tables 1 - 3 and Figures 2 and 3.

PARP Cleavage (cPARP Assay)

The cPARP-FITC fluorescence intensity in the mature fraction was higher in the STS-only group compared with the H_2O_2-only group (Figure 2B)**.** Higher fluorescence intensity in cPARP^{+ve} staining was seen in immature sperm in the STS-only group compared with the H_2O_2-only or control groups (Figure 2C). Exposure to the PARP inhibitor 3-ABA decreased the cPARP-FITC fluorescence intensity in the STS-only group compared with the H_2O_2-only groups (Figure 2D).

Figure 3 TUNEL %+ve in mature **(A)** and immature spermatozoa after chemical or oxidative stress-induced damage in presence or absence of PARP inhibitor; cPARP %+ve in mature **(C)** and immature **(D)** spermatozoa following chemical and oxidative stress-induced damage in presence or absence of PARP inhibitor (STS= staurosporine).



DNA Fragmentation by TUNEL Assay

Immature and mature sperm exposed to STS or H_2O_2 alone or in the presence of 3-ABA did not exhibit a significant difference in the percentage of $TUNEL^{+ve}$ sperm compared with the control group (Figure 3C and 3D).

We next combined the immature and mature sperm groups to examine the effect of 3-ABA on the percentage of $TUNEL^{+ve}$ sperm. A higher but non-significant percentage of $TUNEL^{+ve}$ sperm were seen in both STS + 3-ABA versus STS-only groups and H_2O_2 + 3-ABA-treated vs. H_2O_2-only groups (Table 1; Figure 3 C and 3D).

Table 1 Measured parameters in different modulation treatments without and with PARP- inhibitor (3-ABA)

Parameter	Mean ± SD Median (IQR) (n = 16) [%]	p- value[a]
TUNEL $^{+ve\%}$		
STS Only	12.2 ± 7.2 9.0 (6.7-18.3)	0.52
STS + 3-ABA	11.6 ± 7.9 10.8 (4.1-16.3)	
H_2O_2 Only	11.1 ± 6.9 8.8 (5.6-17.5)	0.42
H_2O_2 + 3-ABA	12.2 ± 9.4 10.2(4.7-18.6)	
c-PARP $^{+ve\%}$		
STS Only	48.3 ± 34.9 46.4 (15.0-85.4)	0.34
STS + 3-ABA	44.3 ± 28.7 49.2(14.7-69.2)	
H_2O_2 Only	47.5 ± 28.2 57.3(19.2-66.4)	0.82
H_2O_2 + 3-ABA	48.7 ± 29.1 50.3(22.6-70.1)	
Late apoptotic sperm $^{+ve\%}$		
STS Only	34.7 ± 21.8 30.2(21.2-46.9)	**< 0.001**
STS + 3-ABA	75.5 ± 16.9 79.1(63.4-91.3)	
H_2O_2 Only	90.9 ± 22.9 96.7(94.2-98.7)	0.55
H_2O_2 + 3-ABA	88.9 ± 24.7 97.4(95.3-98.2)	
Early apoptotic sperm $^{+ve\%}$		
STS Only	8.0 ± 5.1 8.6(2.6-13.1)	0.10
STS + 3-ABA	4.9 ± 4.7 3.7(1.4-6.4)	
H_2O_2 Only	0.62 ± 0.55 0.64(0.10-0.91)	**0.036**
H_2O_2 + 3-ABA	0.93 ± 0.81 0.66(0.33-1.47)	

Apoptosis by Annexin V/ PI assay

Early Apoptosis Early apoptosis was detected by annexin V+ve staining of immature and mature sperm. Exposure to STS only or in combination with 3-ABA did not show a significant difference in the percentage of early apoptotic sperm in both immature and mature sperm compared with the control group. When both immature and mature sperm were combined and examined for effects of STS + 3-ABA exposure, a 38% decrease in early apoptotic sperm percentage was seen compared with the STS-only group; however, this decrease was non-significant (Table 1).

Exposure to oxidative stress by H_2O_2 caused a significant decrease in the percentage of early apoptotic sperm in immature and mature sperm (0.91% ± 0.58%; p = 0.039 and 0.33% ± 0.36%; p = 0.008) compared with the control group. Exposure to H_2O_2 + 3-ABA resulted in a significant increase in the percentage of early apoptotic sperm in immature (1.28 ± 0.39 %; p = 0.039) and mature sperm (0.59 ± 0.52 %; p = 0.008) compared with the control group. When both immature and mature sperm were combined, a significant increase was seen in the percentage of early apoptotic sperm exposed to H_2O_2 + 3-ABA vs. the H_2O_2-only group (p = 0.036; Table 1).

Late apoptosis Immature sperm showed a higher incidence of late apoptotic sperm in the control, STS-only, and STS + 3-ABA groups compared with the mature sperm fractions. A significantly higher percentage of late apoptotic sperm was seen in the immature sperm in the STS-only group (P = 0.039; Table 1). Within the immature and mature sperm fractions, no difference was seen in the percentage of late apoptotic sperm in the STS-only group, while a significantly higher percentage was seen in the STS + 3-ABA- treated group in immature (P = 0.016) and mature (P = 0.023) sperm fraction compared with the control group (Figure 4).

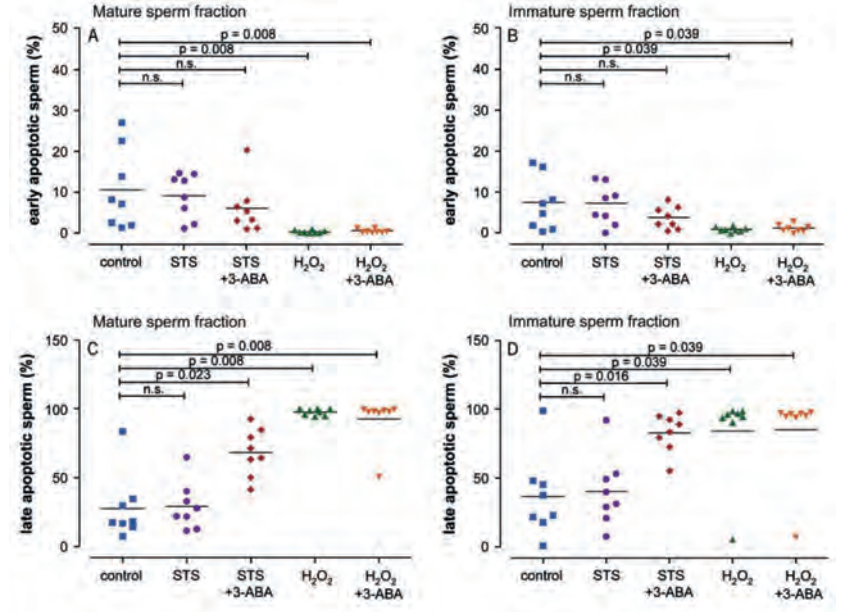
Mature sperm fraction showed a higher incidence of late apoptosis following exposure to both H_2O_2 only and H_2O_2 + 3-ABA compared with immature sperm (P = 0.016 and 0.014, respectively). Within immature and mature fractions, the immature fraction showed a higher incidence of late apoptotic sperm in the H_2O_2-only and H_2O_2 + 3-ABA-exposed groups (P = 0.039 and P = 0.038; respectively; Fig. 4) compared with the control group. Similarly, a higher incidence of late apoptotic sperm was seen in the mature fraction for both H_2O_2-only and H_2O_2 + 3-ABA-exposed groups compared with the control group (P = 0.008 and P = 0.008 respectively; Figure 4).

Combined immature and mature sperm fractions and STS + 3-ABA treatment showed a significantly higher incidence of late apoptotic sperm (P <0.001; Table 2) compared with STS-only groups. Exposure to H_2O_2 + 3-ABA showed a non-significant decrease in the percentage of late apoptotic sperm compared with the H_2O_2-only group (Table 2).

Caspase Activation by Active Caspase-3 Assay

No significant changes in active caspase-3 level were seen in either immature or mature sperm exposed to STS only or H_2O_2 only or in the presence of 3-ABA.

Figure 4 Early apoptotic changes (annexin V +ve) in mature (A) and immature (B) sperm fractions following chemical and oxidative stress-induced damage in presence or absence of PARP inhibitor.



Correlation between cPARP, TUNEL, apoptosis, and active caspase- 3

Apoptotic markers (cPARP, TUNEL, early and late apoptosis, and active caspase-3) were examined for their association with chemical (STS-only and STS + 3-ABA) or oxidative stress (H_2O_2-only and H_2O_2 + 3-ABA) treatment (Table 2). Significant correlations were seen with cPARP in all treated groups (Table 2).

In the untreated group, a positive correlation (r = 0.56, P = 0.02) was observed between the percentages of sperm positive for cPARP and those that were positive for active caspase-3 in the combined (mature and immature) sperm fraction. This correlation became stronger in the STS-only and STS + 3-ABA-treated groups (r = 0.72, and 0.74; P = 0.002, and P <0.001, respectively). In the immature fraction, the percentage of $cPARP^{+ve}$ sperm showed a positive correlation with active $caspase\text{-}3^{+ve}$ sperm in STS-only (r = 0.74; P = 0.046) and STS + 3-ABA (r = 0.81; P = 0.022). Similarly, in the mature fraction, the percentage of $cPARP^{+ve}$ sperm showed a positive correlation with active caspase-3+ve sperm in the STS + 3-ABA-treated (r = 0.83; P = 0.015), H_2O_2-only (r = 0.74; P = 0.046) and H_2O_2 + 3-ABA (r = 0.73; P = 0.046) groups.

Table 2 Correlation between each measured parameter in different modulation treatments without and with PARP inhibitor (3-ABA)

Treatment	Untreated sperm fraction	
	r	P
Activated Caspase-3 $^{+ve}$ spermatozoa		
STS Only	0.94	**<0.001 ^{S}**
STS + 3-ABA	0.98	**<0.001 ^{S}**
H_2O_2 Only	0.95	**<0.001 ^{S}**
H_2O_2 + 3-ABA	0.42	0.10
TUNEL $^{+ve}$ spermatozoa		
STS Only	0.29	0.28
STS + 3-ABA	0.68	**0.004**
H_2O_2 Only	0.64	**0.008**
H_2O_2 + 3-ABA	0.64	**0.007**
Cleaved PARP $^{+ve}$ spermatozoa		
STS Only	0.91	**0.0001**
STS + 3-ABA	0.88	**0.001**
H_2O_2 Only	0.82	**0.001**
H_2O_2 + 3-ABA	0.83	**0.001**
Early apoptotic sperm (annexin V only $^{+ve}$ spermatozoa)		
STS Only	0.72	**0.002**
STS + 3-ABA	-0.13	0.63
H_2O_2 Only	-0.48	0.06
H_2O_2 + 3-ABA	-0.20	0.46
Late apoptotic sperm (annexin V/PI $^{+ve}$ spermatozoa)		
STS Only	0.63	**0.009**
STS + 3-ABA	0.37	0.16
H_2O_2 Only	-0.28	0.29
H_2O_2 + 3-ABA	-0.13	0.63

Discussion

We have demonstrated detectable levels of cPARP in ejaculated human mature and immature spermatozoa by flow cytometry. Our present study puts to rest the controversial debate on the presence [20, 53] or absence [25, 68] of PARP.

Poly(ADP-ribose) polymerase is involved in gamete differentiation during spermatogenesis [51], spermiogenesis [52] and sperm chromatin remodeling [49].

Our findings are in agreement to those of Maymon *et al.* (2006), who reported increased PARP-1 expression in all types of spermatogonia in different maturation stages. These investigators suggested that increased PARP-1 expression may act as a reservoir for maintaining DNA integrity during germline differentiation. Our earlier study [54], in which we reported the presence of PARP homologues in mature and immature spermatozoa, supports this observation. Poly(ADP-ribose) polymerase cleavage may be involved in the DNA damage/ repair process as its incidence is seen to increase with progression of spermatogenesis [48]. The higher, although non-significant, levels of cPARP reported in our study in the mature sperm fraction is supported further by the increased band density of PARP homologues that we previously reported for the first time in mature sperm of proven fertile donors [54]. Our present study findings indicate a potential role of PARP during sperm maturation and the sperm chromatin re-modulation processes. This observation is also supported by several other recent reports [49, 69, 70].

Poly (ADP-ribose) polymerase 1 plays a role in both necrotic and apoptotic cell death pathways as illustrated in Figure 1. Hyper-synthesis of PAR by PARP-1 in response to extensive DNA damage causes depletion of NAD and ATP leading to energy failure and cell necrosis [71]. Poly(ADP-ribose) polymerase 1 can promote a caspase-independent apoptotic pathway through apoptosis inducing factor [72]. Although the mechanism underlying the choice between apoptosis and necrosis in response to genotoxic stimuli is unclear, it may be affected by cell type, stimuli type and strength, and duration of exposure [73].

The present study findings support the above hypothesis of the cell death pathway(s). In the absence of PARP inhibition, exposure to STS-only resulted in a large increase in the percentage of early apoptotic sperm. This increase in early apoptotic sperm with STS declined following PARP inhibition. Furthermore, we obtained a > 2-fold increase in the percentage of late apoptotic sperm following PARP inhibition in STS-induced sperm injury. The increase in late apoptotic sperm with chemical exposure and PARP inhibition are more apparent in immature than in mature sperm fractions, suggesting mature spermatozoa are more protected from chemical-induced damage. This may explain the possible therapeutic application of PARP in selectively facilitating tumor cell death alone or in combination with chemotherapy [74-76] or viral infection [77, 78].

On exposure to oxidative stress, PARP inhibition caused a significant increase only in the percentage of early apoptotic sperm (Table 1). Immature spermatozoa show more resistance to this damage compared with mature sperm, suggesting that mature sperm are more susceptible to oxidative stress. The oxidative stress-induced increase in early apoptotic sperm following PARP inhibition also suggests that over-activation of PARP can increase late apoptosis and necrosis. This is also evident by reports of DNA breaks following oxidative stress causing over-activation of PARP and promoting cell dysfunction/ necrosis in diabetes mellitus, stroke, and cardiovascular diseases [74, 79].
The present study findings show cPARP to be correlated with activated caspase-3. PARP cleavage is catalyzed by activated caspase-3 that initiates apoptosis and prevents PARP-mediated DNA repair processes. Inactivation of PARP has been proposed to prevent depletion of NAD (a PARP substrate) and preserve ATP [80]. Earlier reports have documented apoptosis via oxidative stress [81] and chemical induction [82] through caspase-dependent and independent pathways. One of the mechanisms through which oxidative stress or TNF-alpha mediates cell death is activation of transient receptor potential cation channel, subfamily M, member 2 (TRPM2). This results in increased intracellular Ca^{++} followed by caspase activation and PARP cleavage [83].
Spermatocyte function is reported to be affected by PARP-1 or PARP-2 automodification [84].We hypothesized that sperm with reduced levels of cPARP might be deficient in post-testicular protection/ repair during exposure to a sperm damaging agent. This PARP-dependent mechanism may be inefficient in infertile men; hence, the sperm are more susceptible to damage. In our earlier study, we identified the presence of different PARP homologues in ejaculated sperm [54]. PARP homologues have a variety of molecular and cellular functions. PARP-1 and PARP-2 are involved in DNA repair mechanisms [39, 40, 52, 73, 85].
Collectively, mature and immature spermatozoa showed a significant decline in the percentage of late apoptotic sperm following PARP inhibition in chemical- and oxidative stress-induced sperm damage. The response of oxidative stress-induced damage to PARP inhibition was low compared with that of chemical damage. This suggests that oxidative stress-induced sperm damage may have different pathway(s) such as peroxidation of lipids and proteins in addition to DNA damage. This was evident from the increase in early apoptotic sperm seen in oxidative stress-induced damage even after PARP inhibition.
Our study limitation was the small sample size; however, this was a pilot study. We recommend additional studies with a large sample size. In addition, it will be important to examine DNase- induced injury to ensure direct DNA damage of sperm following exposure to other injurious agents. Optimizing the duration and concentration of exposure to these toxic agents/ PARP inhibitors is also important. Furthermore, it would be interesting to examine the type of PARP homologue cleaved by these treatments, thus opening new therapeutic applications for infertile patients. As we

reported in our earlier study PARP-2 may play a role in sperm damage response and additional studies are needed to explain role of PARP-9 [54].

In conclusion, cPARP is a new apoptotic marker that can be detected in ejaculated spermatozoa by flow cytometry and may be related to the activation of caspase-3. Both immature and mature sperm show cPARP expression following chemical- or oxidative stress-induced damage. PARP inhibition can modulate the incidence of early or late apoptosis in ejaculated human spermatozoa.

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Chapter 4

Single nucleotide polymorphism (SNP) of endothelial nitric oxide synthase (eNOS) gene (Glu298Asp variant) in infertile men with asthenozoospermia

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Abstract

Objective(s): To elucidate the missense GLU298ASP polymorphism within exon 7 of endothelial nitric oxide synthase (eNOS) in infertile men with asthenzoospermia and their potential contribution to sperm motility.

Methods: In this prospective controlled study conducted at our Andrology unit, we investigated the frequency of G894T polymorphism (Glu298Asp variant) within exon 7 of the eNOS gene in 70 infertile men and in 60 healthy men. Sperm motion kinetics were assessed with computer assisted semen analysis (CASA). A single nucleotide polymorphism (SNP) of the eNOS gene in exon 7 (NCBI SNP cluster ID: rs1799983; genbank accession: NG 011992; Protein accession NP 000594) was determined by allele-specific polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP). Sequencing analysis was used to confirm the specific genotype.

Finding(s): The G894T eNOS (T) allele was found at a higher frequency in the patients with asthenozoospermia (60%) compared to the control group (22.5%) (P = 0.02). In those men, the percentage of rapid motile sperm (grade a+b) was low in asthenozoospermic infertile men harbor homozygote's eNOS (TT) genotyping vs.those with wild-type eNOS (GG) (p = 0.02) or heterozygote's eNOS (GT) genotyping (p = 0.01). In the fertile men, the percentage of rapid motile sperm (grade a+b) was higher in the wild-type eNOS than in the eNOS (TT) (p=0.03) or eNOS (GT) genotyping (p=0.04).

Conclusion(s): Our findings suggest that the T allele encoding for aspartic acid of the eNOS (Glu298Asp) gene may contribute to low sperm motility.

Key Words: eNOS, SNP, male infertility, asthenozoospermia, NO, (Glu298Asp) variant.

Introduction

Advanced technologies increase the interest of genetic disorders in infertile men [1]. Nowadays, there is an increased demand and use of various assisted reproduction techniques (ART) such as intracytoplasmic sperm injection (ICSI). In addition, ICSI increased our awarenss about potential genetic risks which may increase the incidence of hereditary genetic causes of transmission infertility or other genetic disorders in the offspring [2, 3].

Oxidative stress (OS) is one of the major issues associated with impaired sperm motility, male infertility and ART outcome [4, 5]. Nitrogen monoxide (·NO), commonly referred to as *nitric oxide* in the biochemical literature [6], is a highly reactive free radical gas generated in biological systems that has a variety of functions. NO is a messenger in a wide array of biological processes [7, 8]. In addition, NO serves as a neurotransmitter in the nervous system as well as a mediator of endothelium-dependent relaxation; it also mediates both tumoricidal and bactericidal actions of macrophages [9, 10]. It has been suggested that NO modulates sexual and reproductive functions in mammalian species [11-13]. Lewis et al. (1996) reported for the first time that NO is synthesized by the human male gamete. Experimental evidence demonstrates that although excessive NO concentrations can cause defective sperm function, low and controlled concentrations of NO play an important role in the control of sperm physiology [14].

Nitric oxide synthase (NOS; EC 1.14.13.39) enzymes produce NO by catalysing a five-electron oxidation of a guanidino nitrogen of L-arginine (L-Arg) [6]. In mammals, 3 distinct genes encode for NOS isozymes; neuronal (nNOS or NOS 1, 150 KDa on chromosome 12, 12q24.2-q24.31); cytokine inducible (iNOS or NOS 2, 130 KDa positioned on chromosome 17, 17q11.2-q12) and endothelial (eNOS or NOS 3), 135 KDa [15]. Balligand et al. (1993) [16] assigned the NOS3 gene to chromosome 7 and regionalized it to 7q35-q36. eNOS and nNOS are isoforms of constitutive NOS and they are involved in cellular signaling pathways [17]. Both enzymes are calcium/calmodulin dependent and they are rapidly activated by agonists that elevate intracellular free Ca^{2+}[18]. Whereas iNOS is a Ca^{2+} independent inducibile NOS isoform [9], iNOS and nNOS are soluble and found predominantly in the cytosol whereas eNOS is a membrane-associated enzyme.

Spermatozoa are the main source of NO. A constitutive NOS appears to be involved in sperm motility, capacitation, and acrosome reaction [19-21]. Using indirect immuno-cytochemistry, the eNOS protein has been localized in the cytoplasm of Leydig, Sertoli cells and all stages of spermatogenesis as well as to the epithelium of the epididymis and vas deferens [22, 23]. .

A single nucleotide polymorphism (SNP) of the eNOS gene within exon 7 includes a G to T mutation at nucleotide position 894 of the eNOS cDNA, which causes glutamic acid to be replaced by aspartic acid at codon 298 (Glu298Asp) during translation of the eNOS enzyme. At present, this variant is associated with both coronary spastic

angina and myocardial infarction [24-26]. There is a lack of knowledge of information in the literature regarding the association between eNOS genotypes and asthenozoospermia—a link we believe may exist because of earlier observation on the association of seminal NO concentration with sperm motility. Our aim was to examine the eNOS gene SNP in infertile men with idiopathic asthenospermia. We examined the possible association(s) between the eNOS missense mutation (Glu298Asp variant) in infertile patients with asthenozoospermia and sperm motion kinetics.

Patients and methods

Study population

This prospective study was approved by the Institutional Review Board (IRB) of the Polytechnic University of Marche, Umberto I Hospital. All participants provided their written informed consent to participate in this study.

Our study consisted of 70 patients who had been referred to the andrology clinic of the endocrinology division of the Umberto I Hospital, Polytechnic University of Marche, in Italyfor an infertility work-up. All of the patients were considered infertile in that they had a female partner with whom they had engaged in unprotected sexual intercourse for at least 18 months without pregnancy. A control group was enrolled as well. These were age-matched men (n=60), whose female partners had achieved pregnancy within the past 2 years. They also had normal sperm motility and other routine sperm parameters as per WHO guidelines [27].

Inclusion/ exclusion criteria

All subjects enrolled in our study had normal sperm morphology (> 30 %) and leukocytospermia (< $1x10^6$/mL) in their semen specimen. The patients had a sperm concentration > $20x10^6$/mL, forward motility (grade a + b) < 50% and/ or grade (a) < 25% and viability >75%. All patients were negative for Mar-test of antisperm antibodies. Semen cultures were negative for microbial infection, including *Chlamydia* and *Mycoplasma ureoliticum* infections. The patients also had normal serum FSH, LH, testosterone, estradiol, and prolactin levels. There was no evidence of anatomical abnormalities of the genital tract, including varicocele (excluded by scrotal doppler sonography). There was no history of criptorchidism, testicular torsion, or genital tract infection. The following exclusion criteria were applied: presence of systemic diseases (*hypertension*, *diabetes mellitus and hypercholesterolemia*), drug treatment within the last 3 months before enrolment in our study, smoking, alcohol use, drug addiction or occupational chemical exposure. Since no possible causes for reduced sperm motility could be detected, our patients were diagnosed with idiopathic asthenozoospermia.

Sperm evaluation

Two complete semen analyses were performed for the study and control populations according to WHO guidelines [27] to ascertain sperm motility and kinetic characteristics. All of these examinations were performed by the same scientist (E.B.). Briefly, 3 µL of liquified semen was placed into a 20 µm cellVU® chamber (Conception Technologies, La Jolla, CA). Two sides (A and B) were loaded for each specimen with scoring of at least 300 spermatozoa, up to 6 different fields per chamber side. Sperm motility was assessed using computer-assisted semen analysis (CASA). Sperm motion characteristics were analyzed with an automated WLJY-9000 sperm analyzer (CGA Scientific Instruments, Florence, Italy). The following sperm kinetic characteristics were evaluated: sperm velocity distribution (rapid + medium, a+b, %), curvilinear velocity (VCL, µm/s), straight linear velocity (VSL, µm/s) and linearity (LIN, %). CASA was performed using low-to-normal settings and calibrated as follows: 20 frames , 4-20 frames/s, 2 track points for calculation of motility, 8 track points for calculation of velocity, and 0-180 µm/s velocity range.

Determination eNOS genotyping

Genomic DNA was extracted from peripheral blood lymphocytes using the Flexigene DNA kit (Qiagen,Valencia, CA) as per the manufacturer's instructions. Isolated genomic DNA was assessed using a Du-62 Spectrophotometer (Beckman, Fullerton, CA) at 260 nm, which also helped to ensure that our extraction method was reliable. A SNP of the eNOS gene in exon 7 (NCBI SNP cluster ID: rs1799983; genbank accession: NG 011992; Protein accession NP 000594) was identified using polymerase chain reaction (PCR) and sequencing analysis. The specific genotype was identified with PCR followed by restriction fragment length polymorphism (RFLP) using the restriction enzyme *Mbo*I and *Ban*II (New England, Bio Labs, Ipswich, MA) to digest mutant and wild alleles, respectively. Breifly, PCR was performed using 50 µL of PCR mixture Buffer (1X Tris-HCl Buffer,pH 8.55), 1.0 mM $MgCl_2$, 0.8 mM dNTPs, plus 0.75 U of DNA Taq polymerase (Finnzyme Oy, Espoo, Finland), 150 ng of genomic DNA template, and each forward and reverse primers at 0.5 µM).

Thermocycling conditions consisted of an initial denaturation for 5 minutes at 94°C; amplification for 35 cycles (denaturation for 30 seconds at 94°C, annealing for 30 seconds at 62°C, and extension for 30 seconds at 72°C) followed by a final step of extension for 5 minutes at 72°C (2700 Thermal Cycler; Applied Biosystem, Foster, CA). PCR primers were generated to amplify the 248-bp fragment encompassing the G894T variant: forward and reverse primers were 5'-AAGGCAGGAGACAGTGG-ATG-GA-3' and 5'- CCCAGTCAATCCCTTTGGTGCTCA-3', respectively [28]. PCR products were separated on 2.5% agarose gel and visualized under UV light after ethidium bromide staining. The PCR product was digested using 5 U of the proper restriction enzyme at 37 °C overnight. The guanine (G) allele at position 894, results in the presence of

glutamic acid (Q) amino acid at position 298, produces two fragments (163 bp and 85 bp in length) in response to *Ban*II restriction enzyme digestion. The thymine (T) allele at position 894, results in the presence of an aspartic acid (D) amino acid at position 298, produces two fragments (158 bp and 90 bp in length) in reposnse to *Mbo*I restriction enzyme digestion. The restriction digest products were analyzed by electrophoresis on 2.0 % agarose gels.
In some cases, sequence validation using the same primers was performed per the manufacturer's instructions. Sequencing was performed using ABI Prism 310 sequencer (Applied Biosystem, Foster, CA).

Statistical analysis

Statistical analysis was performed using the SAS statistical package (Statistical Analysis System Institute, Cary, NC). The Kolmogorov-Smirnov goodness-of-fit test was used to determine ifthe data were normally distributed. For each biallelic marker, allele frequencies were calculated from genotypes in patients and control groups using the Hardy-Weinberg equilibrium. Deviation from Hardy-Weinberg equilibrium was assessed using the Chi Square test (χ^2). Results are expressed as mean ± SD, and they were compared with Student's *t* test between the patients and controls. Values of $p<0.05$ were considered statistically significant.

Results

Screening for missense mutation (Glu298Asp variant) of eNOS gene

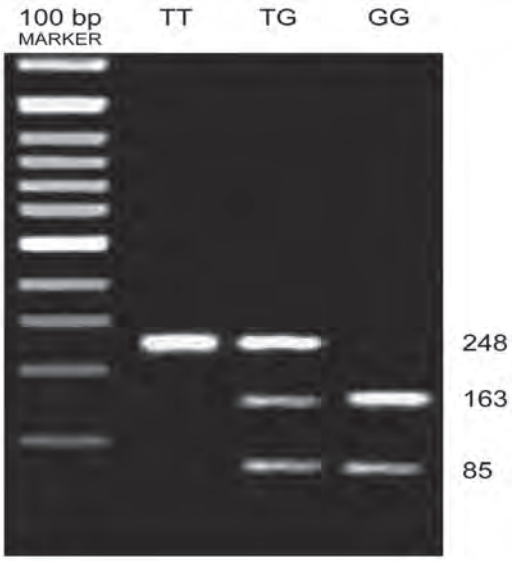
A descriptive comparison of our study populations is presented in table 1. Figure 1 shows agarose gel loaded with 3 PCR products after digestion with BanII enzyme only. Figure 2 (a, b, and c) shows a panel of sequencing pattern analyses of the 3 different genotypes of the eNOS (Glu298Asp) variant. Sequence analysis was done to confirm homozygote, heterozygote of mutant or wild-type genotypes.
eNOS genotypes (TT, GT and GG) were found in 31 (44.3%), 22 (31.4%) and 17 (24.3%) of 70 patients with idiopathic asthenozoospermia, respectively. In contrast, the eNOS genotypes (TT, GT and GG) were found in 9 (15.0%), 9 (15.0%) and 42 (70.0%) of 60 control subjects, respectively. Chi Squared test showed that the genotype frequencies were in agreement with those predicted (not shown) by the Hardy-Weinberg equilibrium (**Table** 1). Additive and dominant effects of the eNOS (T) allele were significantly higher in the patients with idiopathic asthenozoospermia than in the control group, as shown in **Table 1**.

Table 1 Descriptive comparison of age, ejaculate volume, sperm concentration and hormonal levels in asthenozoospermic patients and healthy controls

Clinical parameters	Asthenozoospermic group (n=70)	Control group (n=60)	P value
Age (years)	32.5 (27-38)	40 (30–50)	**>0.05**
Sperm concentration (10^6/ml)	49.55 ± 20.61	55.21 ± 24.69	**0.02**
Ejaculate Volume (ml)	3.2 ± 0.9	2.4 ± 1.1	**0.02**
FSH (mIU/L)	3.8 ± 2.1	2.9 ±1.6	**0.02**
LH (mIU/L)	4.4 ± 2.0	3.7 ± 1.9	**0.02**
Testosterone (ng/ml)	4.7 ± 1.3	4.0 ± 1.5	**0.02**
Estradiol (pg/ml)	31.8 ± 10.4	35.6 ± 12.9	**0.02**
Prolactin (ng/ml)	9.7 ± 4.1	8.8 ± 3.9	**0.02**

Data represented as mean ± SD except for age presented as average (range). Comparison between groups was done by using student t-test. P< 0.05 was considered significant.

Figure 1 A representative agarose gel loaded with three PCR products of (Glu298Asp) homozygote (TT), (Glu298Asp) heterozygote (GT) and wild-type (GG) after digestion with Ban II, respectively. Use of PCR-RFLP analysis to screen for the missense (Glu298Asp) mutation; G to T substitution at nucleotide position 894 of the eNOS cDNA; we used a standard 100 bp marker in this run in the first lane.



Comparison between asthenozoospermic and control groups regarding eNOS gene (Glu298Asp) polymorphism is also shown (**Table 2**). There was a significant increase in allelic frequency of the (Glu298Asp) variants (TT, GT) in asthenozoospermic patient (60%) vs.22.5% for the control groups.

Table 2 Genotype and Allele frequencies for the eNOS gene single nucleotide polymorphism (Glu298Asp variant) in asthenozoospermic patients and healthy controls

	Asthenozoospermic group (n=70)	Control group (n=60)
Genotypes		
eNOS (GG), n (%)	17 (24,29%)	42 (70%)
eNOS (GT), n (%)	22 (31,43%)	9 (15%)
eNOS (TT), n (%)	31 (44,28%)	9 (15%)
χ^2 Test	0,94	0,85
p-value	**0.02**	
Alleles		
G, n (%)	56 (40,00%)	93 (77,50%)
T, n (%)	84 (60,00%)	27 (22,50%)
p-value	**0.02**	

Data represented as number (percentage within group) of patients. eNOS = endothelial nitric oxide synthase; eNOS/GG = homozygous normal; eNOS/TG = heterozygous carriers of the eNOS (Glu298Asp) variant; eNOS/TT = homozygous carriers of the eNOS (Glu298Asp) variant. Chi square (χ^2) test was used to compare the observed frequencies in to those predicted frequencies (not show) by the Hardy-Weinberg equilibrium among patient and control study populations.

Sperm motion kinetics in asthenozoospermic and control subjects groups

We examined the association between kinetic parameters (rapid motile sperm (a+b, %), VCL, VSL, LIN) in the asthenozoospermic patients and controls with different eNOS (Glu298Asp) variants (**Table 3a and Table 3b)**. The comaprisons of sperm kinetic parameters between the control (n=60) and asthenozoospermic groups (n=70) are summarized in Table 4 irrespective to eNOS polymorphism.

In the control group, sperm motion kinetic parameters were significantly different in the men with GG vs.GT alleles regarding rapid motile sperm (a+b, %) (p=0.04) and VCL (p=0.04) and in men with GG vs. TT alleles regarding rapid motile sperm (a+b, %) (p=0.03). On the other hand, asthenozoospermic patients with homozygotes eNOS (TT) alleles showed significantly high values in each sperm motion kinetic parameters (GG vs TT: rapid motile sperm (a+b, %) (p=0.02), VCL (p=0.01), VSL (p=0.02), and LIN

Table 3a Comparison of sperm motion kinetics parameters in infertile men with different genotyping of the eNOS gene among infertile asthenozoospermic men (table 3a) and healthy controls (table 3b)

	Sperm Motion Kinetics			
Genotyping variants	**Rapid Motile Sperm (a+b, %)**	**Curvilinear velocity (VCL, µm/sec)**	**Straight linear velocity (VSL, µm/sec)**	**Linearity (LIN, %)**
eNOS/GG (n=17)	23 ± 15.39	28.58 ± 11.68	13.88 ± 3.05	28.35 ± 8.59
eNOS/GT (n=22)	21.32 ± 8.74	26.54 ± 5.57	12.68 ± 2.21	29 ± 12.19
t-test	0.43	0.72	1.42	-0.19
P Value	>0.05	>0.05	>0.05	>0.05
eNOS/GG (n=17)	23 ± 15.39	28.58 ± 11.68	13.88 ± 3.05	28.35 ± 8.59
eNOS/TT (n=31)	13 ± 12.92	17.71 ± 13.89	10.81 ± 6.26	18.13 ± 12.04
t-test	2.40	2.74	2.28	3.09
P Value	***0.02***	***0.01***	***0.02***	***0.01***
eNOS/GT (n=22)	21.32 ± 8.74	26.54 ± 5.57	12.68 ± 2.21	29 ± 12.19
eNOS/TT (n=31)	13 ± 12.92	17.71 ± 13.89	10.81 ± 6.26	18.13 ± 12.04
t-test	2.62	3.20	1.54	3.22
P Value	***0.01***	***0.002***	***> 0.05***	***0.002***

Data represented as mean ± SD. Student's (t-test) was used for comparison between each two groups. Statistical significance is considered when $p \leq 0.05$. eNOS/GG = homozygous normal; eNOS/GT = heterozygous carrier of eNOS (Glu298Asp) variant; eNOS/TT = homozygous patient of eNOS (Glu298Asp) variant.

Table 3b

Control Subjects	Rapid Motile Sperm (a+b, %)	Curvilinear velocity (VCL, µm/sec)	Straight linear velocity (VSL, µm/sec)	Linearity (LIN, %)
eNOS GG (n=42)	56.81 ± 4.66	48.40 ± 9.89	19.21 ± 3.38	38.47 ± 7.33
eNOS GT (n=9)	53.33 ± 2.82	57.00 ± 15.46	20.55 ± 2.35	41.22 ± 6.24
t-test	2.14	-2.13	-1.13	-1.16
p Value	**0.04**	**0.04**	>0.05	>0.05
eNOS GG (n=42)	56.81 ± 4.66	48.40 ± 9.89	19.21 ± 3.38	38.47 ± 7.33
eNOS TT (n=9)	53.16 ± 3.51	48.77 ± 8.27	19.77 ± 2.58	40.11 ± 6.29
t-test	2.21	-0.11	-0.47	-0.02
p Value	**0.03**	**NS**	**NS**	**NS**
eNOS GT (n=9)	53.33 ± 2.82	57.00 ± 15.46	20.55 ± 2.35	41.22 ± 6.24
eNOS TT (n=9)	53.16 ± 3.51	48.77 ± 8.27	19.77 ± 2.58	40.11 ± 6.29
t-test	0.11	1.41	0.67	0.38
p Value	**NS**	**NS**	**NS**	**NS**

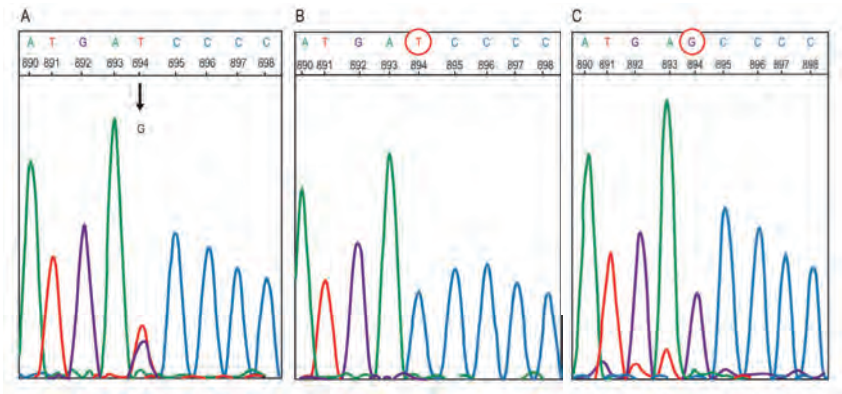
Data presented as mean **±** SD. Student's (t-test) was used for each pair comparison. Significant difference was considered when p≤ 0.05. eNOS/GG = homozygous normal; eNOS/GT = heterozygous carriers of the eNOS (Glu298Asp) variant; eNOS/TT = homozygous carriers of the eNOS (Glu298Asp) variant.

Table 4 Comparison of sperm motion kinetics among infertile asthenozoospermic patients vs. healthy controls

	Control Subjects (n=60) (Means ± SD)	Asthenozoospermic (n=70) (Means ± SD)	p-value
Sperm motility (grade a+b, %)	63.85 ± 7.38	32.52 ± 16.65	**<0.001**
Curvilinear velocity (VCL, µm/sec)	59.55 ± 9.15	35.11 ± 9.69	**<0.001**
Straight linear velocity (VSL, µm/sec)	22.00 ± 1.61	15.42 ± 2.71	**<0.001**
Linearity (LIN, %)	43.29 ± 5.86	33.40 ± 10.57	**<0.001**

(p=0.01). Moreover, the same sperm kinteic parameters were significantly higher in the heterozygotes patients (GT) than in the homozygotes (TT) patients (GT vs TT) regarding rapid motile sperm (a+b, %) (p=0.01), VCL (p=0.002), VSL (p>0.05) and LIN (p=0.002).

Figure 2 Sequencing analysis of the G894T of exon 7 in eNOS gene with different allele expressions, (a) heterozygous (GT) genotyping, (b) mutant homozygotes (TT) genotyping and the (c) wild type homozygotes (GG) genotyping.



Discussion

Spermatozoa are susceptible to oxidative stress (OS) damage. Sperm motility defects are mainly related to the OS damage [29-32]. NO, a short-lived gaseous free radical, has been linked with oxidative stress in various biological systems [33-36]. Our group showed significant high NO concentration in semen from asthenozoospermic infertile comapred to normozoospermic men. Linear negative correlationa between NO concentration with sperm motility has been evidenced, and kinetic characteristics such as VCL and VSL. Furthermore, the eNOS protein expression was higher in asthenozoospermic patients than normozoospermic men [8]. NO synthesized from L-arginine by NO synthases (NOS), is a potent mediator of biologic responses involved in several pathologies. Several eNOS gene polymorphisms have been associated with various vascular, infectious and autoimmune diseases [37].

Middendorff et al.(1997) showed that NOS enzymes and NO receptors are not only present in Leydig cells but also in peritubular lamina propria, Sertoli, and blood vessel cells, suggesting production and activity of NO in these structures [38]. In vitro studies showed that low NO concentrations enhance sperm motility in mouse [20], hamster [21], human [39, 40]. We hypothesized that eNOS gene polymor-phism may be asscoated with abnormal production of NO and oxidative stress (OS) which are associated with male infertility and low sperm quality [4, 8].

In this study, we used the candidate gene approach to explore whether the eNOS encoding gene is involved in sperm motility. For the first time, we describe the association between the eNOS gene and sperm motility and sperm motion kinetics. A nucleotide substitution at the open reading frame causes amino acid substitution of glutamic acid to aspartic acid at a codon in the 298th position. Therefore, we have only limited information about whether this missense mutation gives rise to a functional alteration of eNOS enzymatic activity or if it is a genetic marker associated with some loci. Specifically, we found that there was a statistically significant association between the eNOS (Glu298Asp) SNP and motility in the asthenozoospermic patients. With regard to kinetic parameters, in the asthenozoospermic group, the patients with a wild type (GG) genotype had significantly higher values of sperm motion kinetics than the patients with a heterozygote (GT) or homozygote (TT) genotype except for VSL (Table 3). Recent reports showed involvment of the eNOS gene Glu298Asp polymorphism in various conditions and diseases that are associated with oxidative stress and/ or abnormal NO levels such as attenuation of the vasodilation of in nonexercising muscle [41], advanced stages of endomertiosis [42] and frontotemporal lobar degeneration [24]. Also, Glu298Asp eNOS polymorphism increases the risk ofhypotension in E coli bacteremia [43]. These reports confirm our findings which prove our hypothesis of the possible involvment of Glu298Asp eNOS SNP in abnormal levels of the NO and/ or OS.

In effect, we found a significant difference in the frequency of the missense (Glu298Asp) variant of the eNOS gene between the asthenozoospermic patients and control group. The combined incidence of missense (Glu298Asp) eNOS mutation heterozygote [GT] and homozygote [TT] was 75.71% in the patients with idiopathic asthenozoospermia and 30.0 % in the controls. Thus, our study shows that this missense eNOS polymorphism (Glu298Asp variant) was significantly associated with asthenozoo-spermia and that its homozygote condition (TT) was statistically associated with compromised sperm motion kinetics parameters.

The eNOS missense mutation (Glu298Asp variant) is not located in any functional consensus sequence, but computer analysis revealed that the (Glu298Asp variant) mutation results in a conformational change in the eNOS protein from a helix to a tight turn [44]. The functional significance of this missense (Glu298Asp variant) of the eNOS gene has not yet been demonstrated, suggesting that it may affect the function of eNOS protein. However, X-ray diffraction by protein crystallography is still required to confirm the eNOS 3-dimensional structure, which will provide a better understanding of the molecular basis of the eNOS protein's function. The T allele of this missense eNOS SNP is associated with high plasma NO levels in healthy people [45] and with eNOS protein levels [46]. The eNOS T allele may be associated with an increased production of NO that compromises sperm motility. Presence of T allele may be the cause of altered eNOS protein structure and function which increases the incidence of the OS induced sperm damage in infertile men leading to the asthenozoospermia [47]. The OS induced sperm damage may be related to the abnormal NO levels [8, 40] or due to any other possible associated polymorphism affecting genes responsible for important molecules such as tumor necrosis factor-alpha [1].

Our study findings may one day lead to therapies that inhibit NO activities in patients with low sperm motility. In addition, our study findings may provide novel diagnostic tools for idiopathic male infertility and asthenozoospermia. Additional studies with larger groups of subjects are needed to confirm our findings, to establish a genetic profile that may be useful in the prediction of the outcome of idiopathic infertility, and to establish a relationship with NOS protein activity and NO levels with sperm motility.

In conclusion, we report for the first time a possible role of the missense (Glu298Asp variant) of the eNOS gene in the impairment of sperm motility and kinetic parameters in infertile men. Our findings suggest that the T allele, encoding for aspartic acid, of the eNOS (Glu298Asp) SNP may be associated with low sperm motility.

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Chapter 5

Sperm viability, apoptosis, and intracellular reactive oxygen species levels in human spermatozoa before and after induction of oxidative stress

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Abstract

Objective: To investigate sperm viability, incidence of apoptosis, and intracellular basal and induced reactive oxygen species (ROS) in sperm fractions.
Design: Prospective controlled study.
Setting: Center for Reproductive Medicine at a tertiary care hospital.
Method(s): Liquefied seminal ejaculates (n = 12) prepared by density gradient centrifugation were reconstituted to 2 mL with PBS. Oxidative stress was induced by hydrogen peroxide (H_2O_2; 100μM). Sperm viability, intracellular ROS and incidence of apoptosis/necrosis in neat, immature and mature sperm fractions were assessed.
Result(s): Before H_2O_2 exposure, mature spermatozoa fractions showed significantly lower incidence of apoptotic sperm and intracellular $O_2^{-\cdot}$ levels but higher amounts of intracellular H_2O_2 compared with neat semen. Higher levels of intracellular H_2O_2 were demonstrated in immature sperm fractions compared with neat or mature fractions. In all sperm fractions, intracellular H_2O_2 levels correlated with intracellular concentration of $O_2^{-\cdot}$. Following H_2O_2 exposure, neat semen showed a significantly higher percentage of apoptosis compared with the prepared mature spermatozoa. However, no differences were observed in the incidence of apoptosis between immature and mature sperm fractions.
Conclusion(s): There is a differential shift of both intracellular H_2O_2 and $O_2^{-\cdot}$ in each sperm fractions that may affect sperm quality. Sperm apoptosis is related to intracellular H_2O_2 levels, which in turn are affected by intracellular $O^{-\cdot}$ levels. Oxidative stress was not associated with increased incidence of apoptosis in immature or mature sperm fractions.

Key Words: Reactive oxygen species, flow cytometry, apoptosis, human spermatozoa, intracellular staining.

Introduction

Oxidative stress has been implicated in male infertility [1-5]. Superoxide (O_2–•) and hydrogen peroxide (H_2O_2) are common reactive oxygen species (ROS), which are highly reactive and can interact with nearby molecules, inducing oxidative stress damage in cellular organelles and molecules [6-8].

Physiological levels of ROS are required for normal sperm functions such as hyperactivation, capacitation and acrosome reaction [9-11]. Oxidative stress occurs in spermatozoa when global levels of ROS (both extra- and intracellular) exceed the available total antioxidant capacity. Sperm have a limited amount of cellular cytoplasm where scavenging enzymes are found, making sperm highly susceptible to ROS damage [10, 12, 13]. As ROS are able to readily permeate the membranes, they can cause DNA, proteins and lipid molecules peroxidative damage within the cell [7, 8, 14]. Motile sperm have been shown to be activated by excessive ROS formation and undergo apoptosis-like changes [15-17]. This insult has been linked to sperm apoptosis and male infertility [18, 19].

Sperm preparation plays an important role for successful outcome in assisted reproductive techniques (ART). Double-density gradient centrifugation is a standard sperm selection method for ART to separate mature motile sperm with superior morphology. A mature sperm fraction shows fewer incidences of apoptotic sperm compared with ejaculated unprocessed sperm [20, 21]. Oxidative sperm damage can occur during sperm preparation and processing for ART [22-24].

Oxidative stress-induced sperm damage and apoptosis-like changes may occur when the intracellular ROS levels are in excess of the cells' scavenging capacity [20, 25]. In addition, H_2O_2 has been recommended as a local vaginal contraceptive/spermicidal agent [26, 27]. Measurement of the intracellular ROS would, therefore, be more beneficial than global measurement of the seminal ROS [6, 8, 28]. On the other hand, simultaneous selective measurement of intracellular H_2O_2 or O_2–• levels may be important in understanding how sperm preparation affects ART success rates.

Dichlorofluorescin diacetate (DCFH-DA) and dihydroethidium (DHE) are used for measurement of intracellular H_2O_2 and $O_2^{\bullet-}$, respectively, by flow cytometry. The advantages of using flow cytometry for measurement of intracellular ROS in ejaculated human spermatozoa have been reported recently [29-31].

In this study oxidative stress was induced following exposure to exogenous hydrogen peroxide (H_2O_2). Sperm viability and intracellular ROS levels were evaluated in seminal ejaculates (unprocessed) and in immature and mature sperm fractions to determine the basal and stimulated intracellular ROS levels and examine their relationship with viability and apoptosis.

Material and methods

Sample Collection and Preparation

This study was approved by the Cleveland Clinic Institutional Review Board. Semen samples were collected from 12 healthy male volunteers of unproven fertility status at the Cleveland Clinic andrology laboratory. All samples were collected by masturbation after 2-3 days of sexual abstinence.

After liquefaction, sperm count, percentage motility, viability and presence of round cells were examined on an aliquot of the neat semen sample. The remaining aliquot was prepared for separating mature and immature fractions by double-density gradient centrifugation (PureCeption, SAGE BioPHARMA, Bedminster, NJ). Samples were centrifuged at 300 g for 20 minutes, and the resulting interface between the 40% and 80% layers (immature spermatozoa) was aspirated. Highly motile mature spermatozoa were obtained in 80% pellet. Both fractions were re-suspended in human tubal fluid media (HTF, Irvine Scientific, Santa Ana, CA).

Induction of Oxidative Stress

Oxidative stress was induced by exposing the sperm to H_2O_2. A 30% stock solution of H_2O_2 (Sigma Chemical Co., St Louis, MO) was diluted to 100µM and added to one mL of sperm suspension (stimulated ROS) and incubated for 15 min at 37°C. Another aliquot from the same fraction containing an equal volume of HTF served as control (basal ROS).

Determination of ROS by Flow Cytometry

DCFH-DA, a specific probe for H_2O_2 and DHE, an $O_2^{\cdot-}$-specific probe, are cell-permeable stains. DCFH is oxidized selectively by the free intracellular H_2O_2 into DCF that binds to DNA and emits green fluorescence. HE is oxidized by the free intracellular $O_2^{\cdot-}$ into ethidium bromide that binds to the DNA and emits red fluorescence [31-33]. DCFH–DA (25 µM) and DHE (1.25 µM) (Sigma) were added to the sperm suspension and incubated at 25°C for 40 minutes (DCFH-DA) and 20 minutes (DHE), respectively. Aliquots were subsequently analyzed using a flow cytometer. Green fluorescence (DCF) was evaluated between 500 and 530nm, while red fluorescence (HE) was evaluated between 590 and 700 nm (excitation: 488 nm; emission: 525 - 625 nm in the FL2 channel). Data were expressed as the percentage of fluorescent spermatozoa. Apoptotic spermatozoa were excluded by using counter nucleic acid stains. Propidium iodide (PI) was used as a counter stain dye for DCFH-DA; YO-PRO-1 was used as a counter stain dye for the HE [29].

Apoptosis Detection

To measure the apoptotic status of the spermatozoa, the Vybrant Apoptosis Assay (Invitrogen, Carlsbad, CA) was used. All samples were washed in cold phosphate-

buffered saline (PBS) and the cell density adjusted to ~1 × 10^6 cells/mL in PBS. One µL of the YO-PRO-1 solution (10 µM) and 1 µL of the PI solution (50 µg/mL) were added to 1 mL of cell suspension and incubated for 20–30 minutes. Flow cytometric analysis of the stained cells was done within 1 - 2 h using 488 nm excitation with green fluorescence emission for YO-PRO-1 (i.e., 530/30 bandpass) and red fluorescence emission for PI (i.e., 610/20 bandpass). Gating was performed to exclude any debris. Standard compensation was done using single-color stained controls. Three different populations can be identified by using this assay (Fig 1): viable sperm are negative for both PI and YO-Pro-1, apoptotic sperm are positive for Yo-pro-1 but negative for PI, and dead sperm show positivity for both PI & YoPro-1.

Flow Cytometry Analysis

All fluorescence signals of labeled spermatozoa were analyzed by a Becton Dickinson flow cytometer FACScan (Becton Dickinson, San Jose, CA) equipped with a 488 nm argon laser as a light source. A minimum of 10,000 spermatozoa were examined for each assay at a flow rate of <100 cells/ sec.

The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm supplied by an argon laser at 15 mW. DCF/ YO-PRO-1 emitting green fluorescence and PI/ HE emitting red fluorescence (580–630 nm) were recorded in the FL-1 and FL-2 channels respectively. The percentage of HE/ PI positive cells and the mean fluorescence were calculated on a 1023-channel scale and analyzed using the flow cytometer software FlowJo version 7.2.2 (FlowJo, Ashland, OR).

Statistical Methods

For all quantitative measured parameters, comparisons between stimulated and non-stimulated neat, mature, and immature spermatozoa were performed using the Wilcoxon signed-rank test. Associations among quantitative variables were measured using Spearman's correlation coefficients both within and across sample groups. P value <0.05 was considered as statistically significant

Results

Neat semen samples showed a mean (± SD) semen volume of 2.7 (±1.3) mL, sperm concentration of 62.4 ± 53.1 X 10^6 cells /mL, and motility: 66.6 ± 9.2 %. Before H_2O_2 exposure, mature sperm showed a significantly higher motility (72.7 ± 19.3%) when compared with neat sample (48.9 ± 19.7%, p = 0.014) and immature sperm (41.7 ± 12.3%, p <0.001), respectively.

Sperm Population Identification

When viable, apoptotic, and dead sperm were examined, three different sperm staining patterns were observed upon analyzing the sperm for apoptosis by using the Yo-Pro-1/PI assay (Figure 1 A-F). The apoptotic sperm population stained only positive for Yo-Pro-1. Yo-Pro-1 and PI positive cells represented the dead sperm population, while viable sperm showed negative Yo-Pro-1 and negative PI fluorescence. The percentage of each sperm population in neat, immature, and mature sperm fractions are shown in Figure 1 (A-F).

Sperm Apoptosis and Intracellular ROS before H_2O_2 Exposure:

Density gradient centrifugation selected a population of mature cells with a significantly lower number of apoptotic cells than were present in neat semen ($p = 0.006$) (Table 1). Mature sperm also displayed a higher percentage of DCF $^{+ve}$ ($p < 0.001$) and a lower HE^{+ve} % ($p = 0.004$) fluorescence compared with sperm in the neat semen. Immature sperm showed a significantly higher percentage of DCF-stained sperm when compared with both neat ($p<0.001$) and mature sperm ($p = 0.05$).

Sperm Viability, Apoptosis and Intracellular ROS after H_2O_2 Exposure:

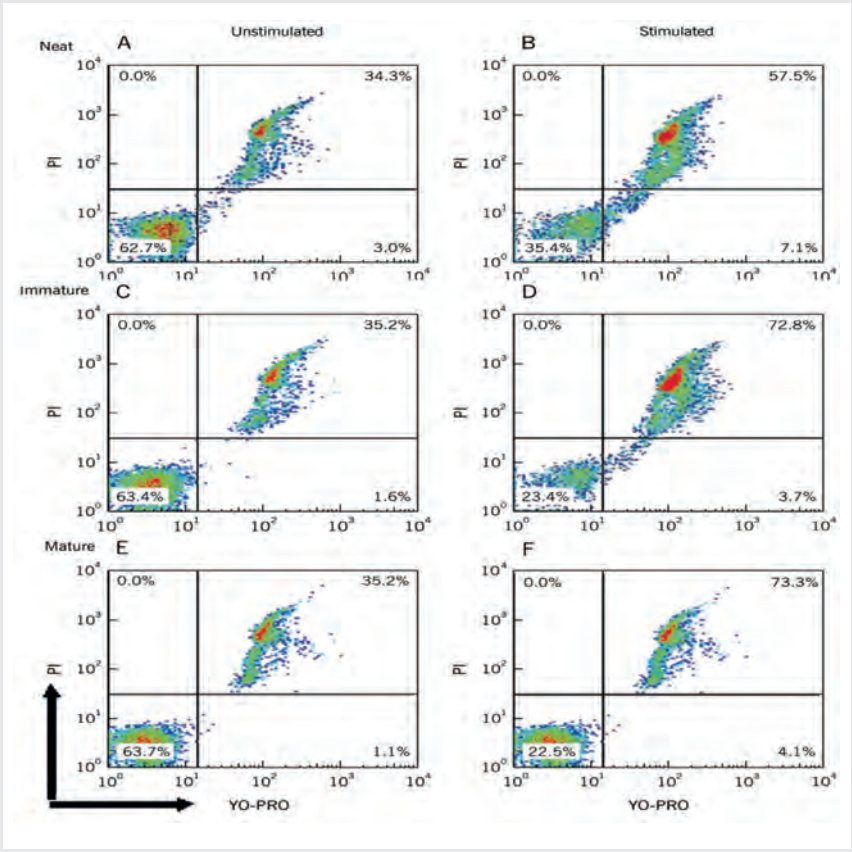
Exposure to H_2O_2 significantly reduced viability and increased the percentage of dead sperm in neat and in immature and mature groups as shown in Figure 1. On the other hand, exposure to H_2O_2 was associated with an increase in the mean percentage of apoptotic sperm in neat semen and in the mature and immature sperm fractions but the difference was not significant (Table 1). Following H_2O_2 exposure, a significantly lower percentage of apoptosis was seen in mature vs. neat sperm fractions ($p = 0.024$). Other comparisons between immature vs. mature and neat (stimulated or non-stimulated) showed no significant differences.

The percentage of sperm with DCF fluorescence (intracellular H_2O_2) increased in both neat and mature sperm, but the difference was not significant in the immature sperm fractions (Table 1) (Fig. 2 A). Moreover, neat, immature and mature sperm fractions exhibited a significant increase in the percentage of sperm showing HE fluorescence (increase intracellular $O_2^{\cdot-}$) (Table1) (Fig. 2B).

Correlation of Intracellular ROS with Other Sperm Parameters

Sperm viability was inversely related to the percentage of apoptotic spermatozoa (mature non-stimulated $r = -0.76$, $p = 0.006$; mature stimulated $r = -0.58$, $p = 0.047$; all fractions; $r = -0.55$, $p < 0.001$). Following H_2O_2 exposure, the percentage of viable spermatozoa was positively correlated with the intensity of DCF fluorescence in neat ($r = 0.63$, $p = 0.031$) and in mature sperm fractions ($r = 0.59$, $p = 0.04$).

Figure 1 Representative flowcytometry pseudo colored dot plots for unstimulated (left) and stimulated (right) **A-B**: neat, **C-D**: immature and **E-F**: mature sperm fractions. Each quadrant is shown as : Lower left quadrant: viable, non-stained sperm; lower right: apoptotic sperm (Yo-Pro positive only); Upper right quadrant: dead spermatozoa (positive for Yo-Pro and PI). The numbers in parenthesis represent the percentage of sperm population in each quadrant. Only 3 sperm population could be identified by using Yo-Pro-1/PI for apoptosis.



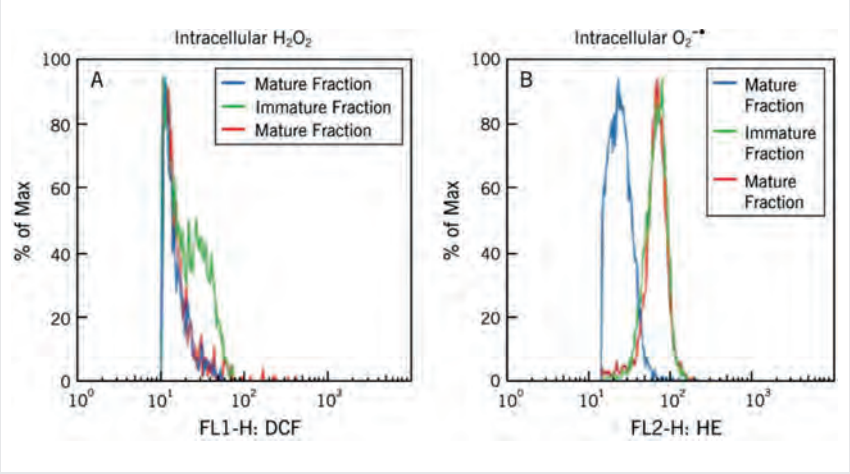
The percentage of apoptotic sperm was positively correlated with DCF fluorescence (intracellular H_2O_2) in neat non-exposed fractions ($r = 0.60$, $p < 0.041$). We wanted to examine the relationship of apoptotic sperm with dead sperm. When the neat, immature and mature fractions (overall) were examined before and after stimulation, the percentage of apoptotic sperm was positively correlated with the percentage of dead sperm in the overall group ($r = 0.52$; $p < 0.001$) and the mature sperm fraction ($r = 0.78$; $p = 0.004$). Following H_2O_2 exposure, the percentage of sperm positive for

Table 1 Comparison of the measured parameters in different sperm fractions before and after H_2O_2 exposure

Marker (ROS assessed)	**Neat semen** Mean ± SD (n = 12)			**Mature sperm** Mean ± SD (n = 12)			**Immature sperm** Mean ±SD (n=12)		
	Non-stimulated	**Stimulated**	**p value**	**Non-stimulated**	**Stimulated**	**p value**	**Non-stimulated**	**Stimulated**	**p value**
Viable sperm (%)	59.4 ± 8.98	46.2 ± 11.9	0.006	67.4 ± 13.4	52.1 ± 19.3	0.08	65.3 ± 9.2	45.8 ± 16.9	0.008
Apoptotic sperm (%)	0.76 ± 0.77	1.7 ± 1.75	0.2	0.25 ± 0.24[a] (0.006)	0.73 ± 0.85[d] (0.024)	0.18	0.41 ± 0..41	1.0 ± 1.15	0.11
Dead Sperm (%)	38.9 ± 10.5	50.3 ± 12.6	0.035	30.9 ± 15.2	46.8 ± 19.0	0.07	33.7 ± 9.2	52.3 ± 16.8	0.009
DCFH-DA % (H_2O_2)	6.4 ± 8.7	38.2 ± 18.2	<0.001	25.3 ± 14.2[a] (<0.001)	50.4 ± 24.1	0.015	36.6 ± 12.0[b,c] (<0.001; 0.05)	50.3 ± 22.6	0.16
DHE % (O_2—•)	3.5 ± 1.8	8.1 ± 5.7	0.024	1.5 ± 1.2[a] (0.004)	16.7 ± 13.1	<0.001	2.1 ± 1.3	9.9 ± 7.3	0.002

[a] = significant difference between non-stimulated mature and neat sperm fractions; [b] = significant difference between non-stimulated immature and neat sperm fractions; [c] = significant difference between non-stimulated mature and immature sperm fractions; [d] = significant difference between stimulated mature and neat sperm fractions. Statistical comparison between stimulated and non-stimulated samples within neat, mature and immature were performed using the Wilcoxon signed-rank test. Pair-wise comparisons of neat, mature and immature within stimulated and non-stimulated samples were also performed using the Wilcoxon signed-rank test.

Figure 2 Representative flowcytometry marker histogram for stimulated neat, immature and mature sperm fractions. A: positive DCF fluorescence (represents intracellular H2O2) B: Positive fluorescence for HE fluorescence (represents intracellular O2−•). The histograms show the differential shift of the intracellular ROS in sperm fractions.



DCF showed a negative correlation with the percentage of dead sperm in neat ($r = -0.68$, $p = 0.01$) and in mature sperm fractions ($r = -0.59$; $p = 0.04$). Similarly, after exposure, the percentage of HE $^{+ve}$ sperm (intracellular $O_2^{-\bullet}$) was significantly correlated with the percentage of DCF $^{+ve}$ sperm (intracellular H_2O_2) in the overall ($r = 0.52$; $p < 0.001$) and mature sperm fractions ($r = 0.78$; $p = 0.004$).

Discussion

Our study aim was to evaluate the basal and stimulated intracellular H_2O_2 and $O_2^{-\bullet}$ levels in different sperm fractions and to examine their relationship with sperm apoptosis. We have measured basal levels of both intracellular H_2O_2 and $O_2^{-\bullet}$ in neat, immature and mature sperm fractions. Interestingly, both mature and immature sperm showed reduced intracellular levels of $O_2^{-\bullet}$ compared with the neat sperm. Higher levels of intracellular H_2O_2 (as represented by DCF $^{+ve}$ fluorescence) were seen in immature compared with mature or neat sperm fractions (Table 1). We report for the first time the shift in intracellular H_2O_2 and $O_2^{-\bullet}$levels. This shift may be explained by the fact that conventional centrifugation increases heat generation, which may affect sperm quality [17, 34, 35]. Avoiding sperm centrifugation for longer times and / or higher speed(s) or its modification may be helpful in preserving sperm quality [36].

We hypothesize that conventional centrifugation may increase the activity of the superoxide dismutase enzyme that converts the generated superoxide ion during centrifugation into hydrogen peroxide, which may prove fatal to sperm cells [37]. This conversion lowers the available intracellular superoxide levels in both immature and mature sperm when compared with the neat unprocessed spermatozoa. However, mature spermatozoa may have higher catalase activity/expression [38] when compared with immature spermatozoa, enabling them to scavenge the generated H_2O_2 more effectively. Interestingly, despite the higher levels of intracellular H_2O_2 seen in the mature sperm fraction, the percentage of dead sperm in this fraction did not increase significantly even though a significant decrease was seen in the percentage of apoptotic sperm. This might be explained by the fact that H_2O_2 is not as lethal as $O_2^{\cdot-}$ for cell viability. Another, more likely, explanation is the fact that mature sperm have the ability to protect themselves against the harmful effects of H_2O_2 exposure [39, 40]. On the other hand, our finding confirms the reports of Donnelly *et al.* [41] that supplementation with ascorbate and alpha-tocopherol in combination protects the sperm from H_2O_2-induced sperm DNA damage by scavenging the ROS generated during sperm preparation.

Our study data demonstrated that mature spermatozoa fractions exhibited lower levels of both apoptotic and dead sperm and displayed higher percentages of viable sperm compared with neat semen (Table 1). These findings were in agreement with earlier studies from our group [30, 42], as well as with other studies where superoxide anions were shown to induce both caspase activation and apoptosis. From these results, it is also worth mentioning that acute exposure of spermatozoa to H_2O_2 results in decreased sperm viability and increased percentage of dead spermatozoa. Apoptosis and necrosis are different death pathways; we examined their relations to sperm viability. This is in agreement with the findings of Conde de la Rosa *et al.* [6] who reported that H_2O_2 induced cell death. It also agrees with others who evaluated the efficacy of H_2O_2 as a local vaginal contraceptive/spermicidal agent with a short time exposure [21, 43-45].

We have demonstrated that intracellular H_2O_2 levels are related to the intracellular $O_2^{\cdot-}$ levels as both are end products of one reaction. Sperm motility is positively related to the percentage of viable sperm. When all the fractions were considered together, motility was inversely related to the percentage of dead or apoptotic sperm. This may explain why sperm motility decreases in pathologically high ROS conditions where there is an increase in the percentage of dead sperm. It may also explain why sperm preparation by density gradient separation shows higher motility due to reducing the percentage of apoptotic spermatozoa. As shown earlier, removal of apoptotic sperm improves the quality of the prepared sperm [42].

Our study findings show that the percentage of apoptotic sperm was positively related to the basal intracellular levels of H_2O_2 in the neat sperm fraction. However,

under high ROS conditions (pathological or induced), intracellular H_2O_2 level was positively related to the percentage of viable spermatozoa in stimulated as well as neat sperm. This may be explained by the fact that induced high ROS conditions will increase the percentage of apoptotic/ dead sperm. In high ROS conditions (pathological or induced), viable spermatozoa may have the ability to adapt to increased H_2O_2 as a result of their high defense status (Figure 1). This observation may be important and supported by the report that shows 100% efficacy (sperm immobilization and loss of viability) in mating studies not earlier than 2 h of H_2O_2 exposure [43]. This may explain the poor efficacy of H_2O_2 as a local vaginal contraceptive for short time usage (<1 h) and is in accordance with a report [43] that 2 hours exposure may be appropriate to improve the H_2O_2 contraceptive efficiency.

In conclusion, both immature and mature sperm produce higher intracellular H_2O_2 levels compared with neat spermatozoa. This probably is attributable to sperm processing with centrifugation. Mature spermatozoa may adapt to H_2O_2 generated during sperm preparation involving centrifugation. This may explain the presence of the higher percentage of viable and the lower percentage of dead/ apoptotic sperm in the prepared mature spermatozoa fraction. Sperm preparation may be associated with differential shift of both intracellular H_2O_2 and $O_2^{-\bullet}$ that may affect the sperm quality. Apoptotic changes in sperm are attributed largely to the intracellular H_2O_2 levels while dead sperm are related to intracellular $O_2^{-\bullet}$ levels. Finally, intracellular ROS levels may affect sperm quality through their effects on sperm viability**.**

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Part III

Applications of Apoptotic Markers in Andrology Lab

Chapter 6

Diagnostic value of the total antioxidant capacity (TAC) in human seminal plasma

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Abstract

Objective: To establish cutoff value, sensitivity, specificity and intra- and inter-observer variability of total antioxidant capacity (TAC) in seminal plasma from healthy donors (controls) and infertile patients.
Design: Seminal plasma from proven fertile donors (n = 55), non-proven fertile donors (n = 45) and infertile patients (n = 42) were examined for TAC level
Settings: Reproductive research center in a tertiary care hospital.
Patient(s): Infertile patients from male infertility clinic of various diagnoses
Intervention(s): Seminal plasma TAC measurement by a colorimetric assay using the TAC assay kit, receiver operating curve.
Main Outcome Measure(s): Seminal plasma TAC levels, cutoff value, sensitivity, and specificity.
Result(s): Proven fertile donors showed higher TAC values (median and range): 1700 (1440 - 2290 μM); compared to the infertile patients: 1310 (1040 - 1600 μM). The best cutoff to distinguish between fertile controls and infertile men was 1420 μM. At this threshold, specificity was 64% and sensitivity 76%.
Conclusion(s): Total antioxidant capacity of the seminal plasma as measured by the colorimetric assay is a reliable and simple test for the diagnosis and management of male infertility.

Key words: Seminal plasma, male infertility, oxidative stress, receiver operating characteristic curve, total antioxidant capacity.

Introduction

Male factor infertility accounts for 30 to 50% of the total infertile couples seeking for infertility management [1]. Among the couples seen for infertility, the most common cause of male infertility is defective spermatozoal function. It may result from testicular pathologies, genetic disorders, and exposure to drugs, toxins or irradiations, or because of oxidative stress damage [2, 3]. The mechanism of action for loss of sperm function may be due to elevated levels of reactive oxygen species (ROS) beyond the available total antioxidant capacity in the semen [3-9].

Seminal plasma has a very effective antioxidant systems that can provide the spermatozoa with a protective environment against oxidative stress [10]. This protection compensates for the loss of cytoplasmic sperm enzymes that occurs during maturation and transportation processes, which in turn diminishes the spermatozoa's endogenous enzymatic and repair defenses [7, 8, 11-15]. Indeed, the total antioxidant capacity of seminal plasma is due to the sum of enzymatic (e.g. superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic (e.g. ascorbate, urate, vitamin E, pyruvate, glutathione, taurine, and hypotaurine) antioxidants [7, 16-21].

Low level of seminal total antioxidant capacity (TAC) has a key role in male infertility [8, 22, 23]. It is important to ensure that any measurement of seminal TAC is accurate and reliable and yet easy to use as a diagnostic tool in the evaluation and follow-up of male infertility. TAC levels have been measured by the enhanced chemiluminescence method earlier in patients with clinical diagnosis such as varicocele, varicocele with infection, vasectomy reversal as well as idiopathic infertility [9, 24, 25]. In addition, using the ROS and TAC results, a novel score called the ROS-TAC score was also described earlier by us in subsets of patients with various clinical diagnosis[1, 16, 25-27]. This score was described as a better predictor of oxidative stress in patients with various clinical diagnosis compared to ROS or the TAC values alone. Seminal TAC can be measured as the total available antioxidant protection in the seminal plasma which is more practical and easy to perform by an enhanced chemiluminescence or the colorimetric assay. In contrast, measuring specific antioxidant assays are expensive and cumbersome in performance (High pressure liquid chromatography, and so forth) and provide limited information about the assessed antioxidants. Earlier we compared the enhanced chemiluminescence and colorimetric method to assess TAC. We reported that the colorimetric TAC measurement is simple, rapid, cheaper and accurate [28].

Increase in levels of ROS without a concomitant rise in antioxidant defenses leads to oxidative stress. Oxidative stress is involved in the pathogenesis of male and female reproduction. Oxidative stress causes damage to the spermatozoa, oocyte and embryos. Several reports relate low seminal plasma TAC levels to male infertility as well as in embryo culture media from the oocytes, cumulus cell mass and spermatozoa used for insemination in conventional IVF. The potential cellular sources of TAC in ICSI

setting are the spermatozoa and the injected oocytes [8, 22, 23, 29-36]. However, a definite cutoff or reference range in infertile patients or proven fertile subjects for a possible use as a diagnostic tool for infertility identification is lacking.
The objectives of our study were to [1] identify a cutoff value for seminal plasma TAC level that can differentiate infertile patients from fertile donors, [2] establish the sensitivity and specificity of the test; and [3] examine the intra- and inter-observer variability of the assay to establish the variability between various individuals who may perform the assay in a clinical laboratory setting.

Materials and methods

Subject Selection

This study was approved by the Institutional Review Board of our hospital. Infertile patients (n = 42) as well as normal healthy men (n = 100) were screened and selected on the basis of normal semen analysis according to the World Health Organization (WHO) [37] guidelines.

Semen Collection and Preparation

Semen specimens were collected by masturbation after 48 to 72 hours of sexual abstinence. The specimens underwent complete liquefaction at 37°C for 20 minutes, and 5 µL of each specimen was loaded on a 20 µL Microcell chamber (Conception Technologies, San Diego, CA) where it was analyzed for sperm concentration and motility. Samples were classified according to the results of semen analysis as per the WHO (1999) [37] criteria and history of establishing a pregnancy in the past 2 years.
Donors or controls (n = 55) were further classified as proven fertile that is, who had initiated a pregnancy within the past 2 years and had normal semen analysis results according to WHO criteria. Unproven fertile (n = 45) men were those who had normal semen analysis results but had not established a pregnancy in the past 2 years. Infertile patient group (n = 42) consisted of men who had abnormal semen analysis and were presenting for infertility treatment and /or undergoing investigation for various male infertility factors. All samples were centrifuged at 1,000 X g for 10 minutes at 4°C. Clear seminal plasma was aliquoted and frozen at -70°C till the time of TAC assay. A total of 142 TAC measures were conducted: 42 for patients, 55 for fertile donors and 45 for donors with unknown fertility status.

Total antioxidant assay

Seminal plasma total antioxidant measurement was done using the antioxidant assay kit (Cat # 709001; Cayman Chemical, Ann Arbor, MI). The Cayman chemical antioxidant assay was used to measure the antioxidant capacity of the seminal fluid samples. The

principal of the assay is the ability of aqueous- and lipid-antioxidants in the seminal plasma specimens to inhibit the oxidation of the 2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to $ABTS^{+}$. Under the reaction conditions used, the antioxidants in the seminal plasma cause suppression of the absorbance at 750 nm to a degree which is proportional to their concentration. The capacity of the antioxidants present in the sample to prevent $ABTS^{*}$ oxidation was compared with that of standard - Trolox, a water-soluble tocopherol analogue. Results are reported as micromoles of Trolox equivalent. This assay measures the combined antioxidant activities of all its constituents including vitamins, proteins, lipids, glutathione, uric acid, and so forth.
The technique for TAC assay used in our study has been described before [38, 39]. All seminal plasma samples were diluted 1:10 with the assay buffer before assaying to avoid variability due to interference by the plasma proteins or sample dilution. All reagents and samples were equilibrated to room temperature before beginning the assay. Samples as well as Trolox standards were assayed in duplicate. Trolox standards and reagent were prepared as per the manufacturer's instructions at the time of the assay. After the plate configuration, 10 µL of Trolox standard and samples were loaded on to the corresponding wells of a 96 well plate. Then 10 µL of metmyoglobin and 150 µL of chromogen were added to all standard/sample wells. The reaction was initiated by adding 40 µL of hydrogen peroxide (H_2O_2) as quickly as possible. The plate was covered and incubated for 5 minutes on a shaker at room temperature. Absorbance was monitored at 750 nm using ELx800™ Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT).

Calculation of assay result

Determination of the reaction rate was done by calculating the average absorbance of each standard and sample. The average absorbance of the standards as a function of the final Trolox concentration (µM) was plotted for the standards curve in each run, from which the unknown samples were determined (Figure 1). The total antioxidant concentration of each sample was calculated using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the equation:

$$\text{Antioxidant } (\mu M) = \frac{\text{Unknown average absorbance} - \text{Y-intercept}}{\text{Slope}} \text{ X dilution X } 1000$$

Precision and sensitivity of the TAC assay kit

According to the manufacturer, the assay precision was: inter-assay coefficient of variation 3% (n = 20) and intra-assay coefficient of variation 3.4% (n = 84). The assay kit could measure samples containing antioxidants ranging between 44 - 330 µmoles without any further dilution.

Interobserver and Intraobserver Variation

For each subject, 1-2 measurements were recorded. Two observers (RM and RS) conducted these measurements both from the same subjects as well as from different subjects.

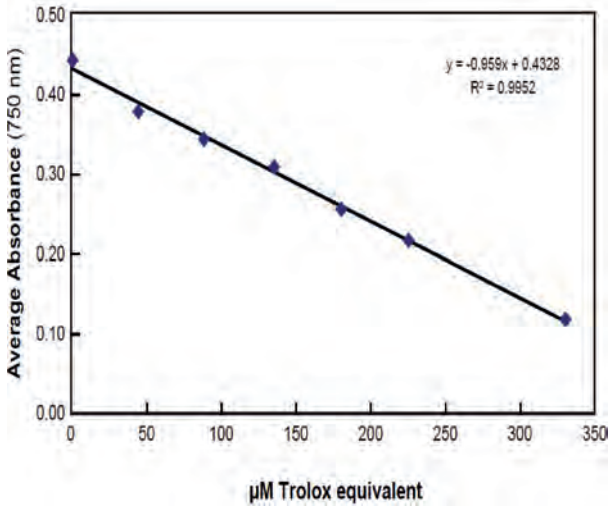
The inter- and intra-observer variability illustrated in Figure 1 was evaluated using a total of 60 TAC measurements. Two observers evaluated 8 donors and 7 patients in duplicate (n = 30 each). The inter observer variation was obtained by analyzing the differences in the results produced by the 2 observers. The intra-observer variation was obtained by analyzing the differences between measurements within each observer.

Statistical Analysis

The difference in distributions of TAC levels between infertile patients and proven fertile donors was assessed using the Wilcoxon rank sum test. Summaries of the distributions include mean, standard deviation, median, and interquartile range (IQR). A receiver operating characteristic curve was used to display estimated sensitivity and specificity over a range of possible cutoff points for TAC as a predictor of fertility. A cutoff value was chosen to maximize the sum of sensitivity and specificity. A secondary analysis was performed in a similar fashion that added donors with unproven fertility to the group of donors with proven fertility for comparison to the infertile patients.

Inter- and intra-observer reliability of TAC measurement was assessed between two selected observers. This was calculated in a set consisting of both donors and patients using each observer's measurement in duplicate. For intra-observer reliability, the absolute difference for each sample between the two TAC measurements, divided by the average of the two (denoted as "d/m"), was used as a measure of relative nearness of the measurements. The frequency in which the relative nearness was within set levels was used to describe the intra-observer reliability for each of the two observers. For inter-observer reliability, the two observer's average measurements for each subject were treated as a pair. Differences (not in absolute value), divided by the averages (denoted as"d/m"), and within the pairs were computed as measures of relative nearness of the two observers' TAC measurements. Frequencies within 20 % were then used to describe inter-observer reliability. A one- sample T-test was used to determine if the mean relative nearness of the two observers' TAC measurements was significantly different from zero, and a 95% confidence interval (CI) for the mean was computed. For both intra and inter-observer reliability, tests based on a Spearman correlation coefficient were used to confirm no evidence of the association between the relative nearness and the measurement magnitudes. All analyses were performed using R version 2.3.1 (www.R-project.org) [40].

Figure 1 A standard curve used for TAC measurement, Trolox standard concentrations are represented on x-axis while absorbance at the y-axis.



Results

Receiver Operator Characteristic Curve Analysis

Proven fertile donors versus. infertile patients We have examined the association between TAC levels and the status of a subject, as either infertile patient or a fertile donor. We first considered only proven fertile donors who had normal semen analysis results and had fathered a child in the last 2 years. The distribution of TAC levels within the proven fertile and donors of unproven fertility is shown in Figure 2 and 3.

The mean ± SD and median (IQR) values were as follows: infertile patients: 1380 ± 430 µM, and 1310 (1040 - 1600) µM; proven fertile donors: 1830 ± 540 µM and 1700 (1440 - 2290) µM (Figure 2). Proven fertile donors showed significant higher seminal plasma TAC levels compared to infertile patients groups ($p<0.001$). The cutoff which maximized the sum of the sensitivity and specificity when using TAC as a predictor of fertility was 1420 µM seminal plasma TAC level. The ROC curve showed 76% sensitivity and 64% specificity for this optimal cutoff (Figure 4).

Figure 2 Boxplot for seminal plasma TAC levels of the infertile patient group vs. proven fertile donors group.

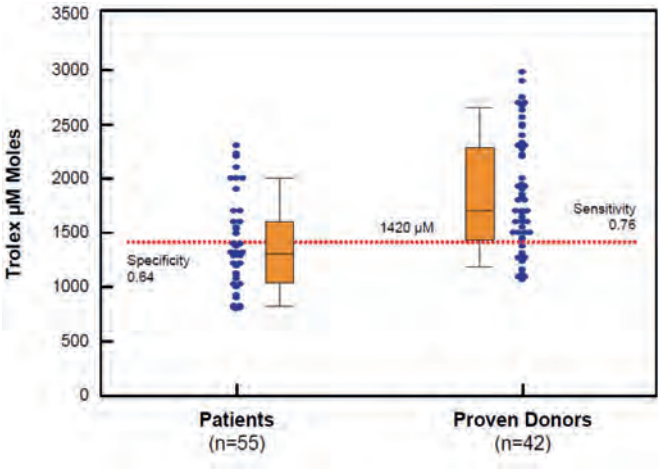


Figure 3 ROC curve to show the best cutoff value with its sensitivity and specificity when used to differentiate infertile patients from proven fertile sperm donors.

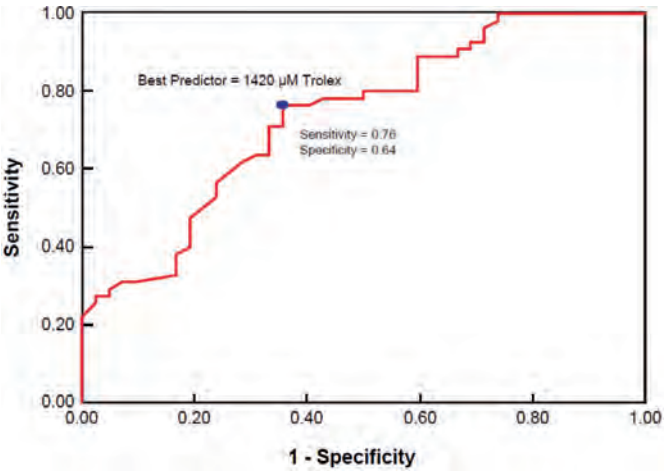


Figure 4 Box plot of seminal plasma TAC levels on the infertile patients vs. all sperm donors group.

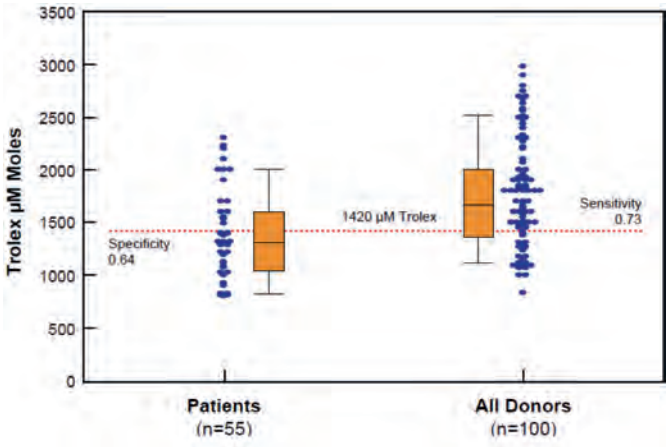
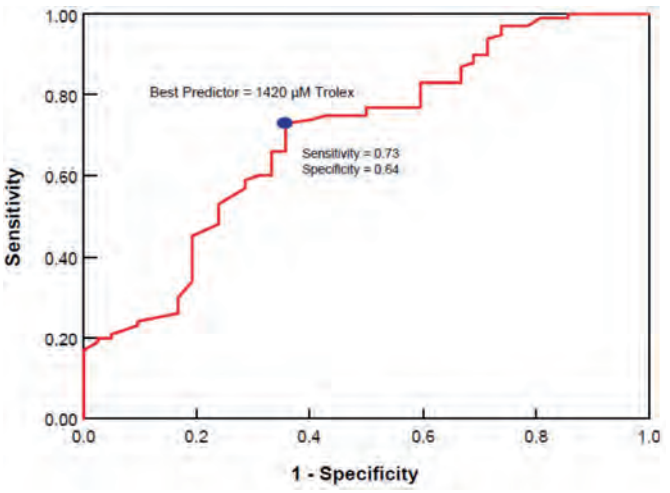


Figure 5 ROC curve is used to predict the best cutoff value of seminal plasma TAC levels of the infertile patients vs. proven fertile sperm donors with sensitivity and specificity.



All donors vs. infertile patients As a secondary analysis, we compared infertile patients to all donors (whether proven fertile or not). The TAC values in all donors (proven and unproven) was 1740 ± 510 µM and 1670 (1360 - 2000) µM versus infertile patients: 1380 ± 430 µM, and 1310 (1040 - 1600) µM; (Figure 3). The results of this analysis were similar to those that exclude the unproven donors. Significant difference in the TAC values was seen in the 2 groups when a similar cutoff value of 1420 µM was selected (Figure 5).

Intra- and inter-Observer variability The intra- and inter-observer differences were calculated using the simple difference rather than a standard deviation. Rather than the CV, we used a ratio of a difference to mean (d/m) to represent the relative difference of two TAC measurements. Next we assessed the likelihood that such relative differences would exceed 20% value which is unacceptable. By using this ratio; the mean intra-observer variability was 13.4% for observer-1 and 15 % for observer-2. The mean inter-observer variability between the two observers was 19 %. All the variability measures were in the acceptable ranges (d/m ratio <20 %).

Discussion

The link between reactive oxygen species and the male infertility has been established. Irrespective of the clinical manifestation and the results of semen analysis, the presence of abnormal ROS level plays a major role in the pathogenesis of male infertility [27, 30, 36, 41, 42].

Total seminal plasma antioxidants are the most protective defensive mechanism available to spermatozoa against ROS. Low seminal total antioxidant capacity has been shown to be related to male infertility [10, 28, 35, 36]. Seminal plasma antioxidant capacity may be influenced by a wide range of the factors such as nutrition, vitamin supply, age, infection, etc), therefore, it is important to accurately estimate the total antioxidant amount of the seminal plasma [19, 38]. From the methods available for measuring TAC, earlier we compared the more commonly used enhanced chemiluminescence assay versus the colorimetric assay using for measuring TAC levels in seminal plasma.

For the colorimetric assay we used the TAC kit from Randox (Randox Laboratories Ltd, San Francisco, CA).The measurement of total antioxidant capacity by enhanced chemiluminescent method generally takes approximately 40-45 minutes; it requires stringent assay conditions as opposed to the colorimetric assay. The price of an average luminometer with kinetic setting averages $30,000 compared with $6,000 for a simple spectrophotometer. We reported that the colorimetric assay is a reliable, simple, rapid, and accurate assay compared with the chemiluminescence assay [28].

The main objective of our study was to examine the diagnostic value of the seminal

plasma total antioxidant capacity (TAC) assay in infertility clinic in terms of the best cutoff level, assay sensitivity and specificity using a colorimetric assay. Also we examined the inter- and intra-observer variability for this assay to establish the variability between various individuals who may perform the assay in a clinical laboratory setting.

It is important that the technicians are checked off for accuracy especially in routine bench work such as accuracy in pipetting, and so forth. In our study the two observers (R.M. and R.K.S.) were very experienced in routine bench work (>5 years). It is prudent to have more researchers validate our study. The variation is near the acceptable limit. This also depends on the method of calculating the variation. Here we calculated the ratio of the absolute difference to the average value. This is different from the coefficient of variation (CV), which is calculated from the ratio of standard deviation to the average. The d/m ratio is more sensitive to the CV. By using the CV calculations, the inter- and intraobserver variability was around 5%.

In our study, infertile patient group showed lower seminal plasma TAC levels compared to the proven fertile and the overall donor group (Figure 2 and 3). The proven fertile and all donors groups showed comparable seminal plasma TAC levels. We have established a cutoff value of 1420 micromoles of trolox; all infertile men showed TAC levels below this cutoff value, while proven fertile men showed TAC levels higher than this cutoff value. Receiver operating characteristic curve showed high sensitivity (76%) and specificity (64%). Inter-observer and intra-observer variation were within the acceptable ranges.

Siciliano et al [21] reported similar total antioxidant status using ABTS based colorimetric assay in asthenozoospermic (1240 ± 200 µmoles) and oligoasthenozoospermic patients (1260 ± 210 µmoles) with normal viscosity compared with normozoospermic healthy donors (1210 ± 160 µmoles) with proven fertility [21]. However significant differences were seen in the TAC levels in these patients with normal viscosity when compared to those that were hyperviscous. They concluded that impaired antioxidants in asthenozoospermic patients may be related to other associated pathologies such as hyperviscosity. Contrary to their findings, low TAC levels were seen in our infertile patient groups and these levels were significantly lower than in proven as well as unproven fertile donors. None of the samples in our study was hyperviscous.

Fingerova et al [8] reported seminal plasma TAC level to be about 1.4 times higher than those in the blood serum [8]. They measured the seminal plasma TAC levels by using Randox kit. They found significantly lower values (mean ± SD 2.15 ± 0.29 mM) in the infertile patients compared to the control 2.32 ± 0.33 mM; P <.02). When the range and median were compared there appeared to be an overlap between the patients and controls (patients: median and range: 2.11 (1.63 - 2.64) mM versus control: 2.48 (1.45 - 2.66) mM. Our results agree with their finding regarding the significantly lower TAC values in patients compared with the controls. However the difference in our

cutoff value between patients and controls is more distinct compared to the values reported in the study by Fingerova et al. This can be attributed to the fact that in their study the patient and control semen analysis results were more or less within normal ranges according to WHO references [37].

Lewis et al [23] compared the individual antioxidants in serum and in seminal plasma. They reported that in seminal plasma ascorbate, urates and thiols are the major antioxidant available. [23]. However; Donnely et al. reported that single or combined supplementation of ascorbate and tocopherol is not beneficial for sperm motility improvement [7]. Their findings suggest the importance of the TAC assay instead of measuring individual antioxidants which is complex & inaccurate. It may therefore be important to use TAC assay for monitoring and follow up for infertile patients.

Our inter- and intraobserver variability results were in agreement with our earlier work where we reported the actual average difference of 27.6 µM Trolox equivalent between observers, and inter-observer variation of 3 % (95%CI; -3.6%-9.5%) [28]. Benzie et al [18] reported intra-assay CV of <1.0% and inter-assay CV of <3.0% using the ferric reducing ability of plasma (FRAP) as a measure of the antioxidant power in the biological fluid samples.

Using the enhanced chemiluminescence assay for measuring TAC levels we demonstrated the usefulness of this assay in identifying patients with various clinical diagnosis [9, 24, 25]. We also defined a novel ROS-TAC score and its usefulness in differentiating the patients from control healthy men [1, 16, 25, 26, 43]. Using the enhanced chemiluminescence assay we reported the TAC values in controls 1650.9 ± 532.22 [1, 16, 27] and 1653.98 ± 115.29 molar trolox equivalents [25]. All infertile patients with various clinical diagnoses had significantly lower levels of TAC. However the enhanced chemiluminescence assay probe is no longer available and the TAC measurement derived from the luminometer are complex and time consuming. The equipment must be simple, easy to use, and cost effective. Although, enhanced chemilumenscence involves the luminometer, many labs also use high performance liquid chromatography (HPLC) for measuring vitamin E and C concentrations. This is again time consuming, requires specific instrumentation, and is expensive.

We therefore sought to look for an alternate, easy and accurate method for measuring the seminal plasma antioxidant status. We demonstrated the ease of using the colorimetric assay (Randox kit) versus the chemiluminescence method for TAC measurement [28]. The accuracy, sensitivity and the specificity was comparable in the two methods. However, this kit is expensive, uses larger assay volumes and can be used only with a spectrophotometer. Our goal was to use an easy and simple colorimetric assay where multiple samples can be evaluated in smaller volumes simultaneously and therefore we looked for alternate assay kits. The assay kit used currently in our study is cost effective, requires smaller assay and reagent volumes and more importantly it can be used in a plate reader for simultaneous measurement

of large number of samples unlike the Randox kit. In the current study, we included patients and donor based on their semen analysis and fertility status only. In this study, our primary objective was to establish a diagnostic reference values for seminal plasma TAC levels in infertile patients as well as in healthy donors, and hence the abstinence period required was 2–3 days similar to that defined by WHO for routine semen analysis as recommended by WHO [37]. It would be interesting to examine the effect of shorter abstinence period of <48 hours, as this may help decrease the amount of time the spermatozoa are subjected to oxidation while in the male reproductive tract and possibly increase the sensitivity. We recommend further studies to not only reproduce our results, but to establish reference values for different age ranges and also with certain diseases such as varicocele and infection, as well as conditions such as hyperviscosity and varying abstinence period.

In conclusion**,** the simple colorimetric seminal plasma TAC assay used in our study can discriminate proven fertile from infertile patients. It may be used as a diagnostic and prognostic tool in the male infertility clinic. The best obtained predictive value of seminal plasma TAC level is 1420 µM. Therefore, we can recommend seminal plasma TAC measurement as a quick in-office test for the evaluation of patients with male infertility.

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Chapter 7

Evaluation of chemiluminescence and flowcytometry as tools in assessing production of hydrogen peroxide and superoxide anion in human spermatozoa

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Abstract

Objective: To examine simultaneously the levels of superoxide ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2) using chemiluminescence and flowcytometry (FACS).
Design: Prospective laboratory study.
..**Setting**: Reproductive research lab in a tertiary hospital...
..**Subjects**: Semen samples from 18 healthy male volunteers.
Interventions: Sperm preparation and measurement of ROS by chemiluminescence using luminol and lucigenin before and after H_2O_2 exposure and by flow cytometery using Dichlorofluorescin diacetate (DCFH-DA) for H_2O_2 and Dihydroethidium (DHE) for $O_2^{-\cdot}$.
Main Outcome Measures: Sperm count, motility, viability and ROS levels.
Results: Immature sperm fractions showed significantly higher levels of ROS measured by either luminol or lucigenin compared to the nat and mature fraction. Luminol and lucigenin showed significant relationships to DCF^{+ve} and HE^{+ve} sperm. ROS levels were detectable by flowcytometery in chemiluminescence negative samples. Both mature and immature sperm fractions had significantly higher % of cells positive for H_2O_2 compared to neat semen ($p< 0.0001$ and $p= 0.0055$, respectively). On the other hand, $O_2^{-\cdot}$ positive cells in neat semen was significantly higher compared to mature fractions ($p=0.0007$) but significantly lower than that in the immature sperm fractions ($p=0.026$).
Conclusions: we recommend ROS measurement by flowcytometry on the basis that it requires a lower sperm count, is comparable to chemilumenscence, and has higher specificity for intracellular ROS in viable spermatozoa. Samples tested by chemilumensce negative by chemiluminescence still may have high intracellular H_2O_2 generation

Key Words: Superoxide, hydrogen peroxide, flowcytometry, chemiluminescence, semen analysis.

Introduction

The influence of oxidative stress (OS) on male fertility has been extensively studied in the last decade [1-3]. Superoxide ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) are the common form of reactive oxygen species (ROS). ROS are highly reactive and short-lived (half-life ranging from a few nanoseconds to milliseconds). They can interact with nearby molecules and thus play a key role in inducing sperm damage [1, 4-7]. ROS damage affects the lipid and protein content of the spermatozoa. Sperm DNA damage by ROS occurs by induction of high frequency of single or double DNA strand breaks. DNA sperm damage can be detected by terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) or sperm chromatin structure assay (SCSA) [8-12]. Both extracellular and intracellular generated ROS are highly diffusible and can produce significant sperm DNA damage beyond the repair capacity of the growing embryo after fertilization [13].

Chemiluminescence using luminol or lucigenin has been the common method to measure ROS [14, 15]. Luminol probe can measure both intra- and extracellular ROS, especially $O_2^{-\bullet}$, H_2O_2, and OH^- free radicals, while lucigenin can detect extracellular ROS, especially $O_2^{-\bullet}$, OH^- free radical. Consequently, both probes provide global ROS values but no detailed information on intracellular levels of H_2O_2 or $O_2^{-\bullet}$ [14, 15].

The chemiluminescence assay has several limitations. These include [1] the need for a relatively high volume and sperm concentration, [2] decline of ROS with time after ejaculation, [3] the interference of iron and copper in the culture media, [4] the inability to detect a specific type of ROS, and [5] the inability to differentiate viable from apoptotic spermatozoa. However, reference values for standard or clinically useful ROS levels are lacking [16]. Recently, we reported [17] the cutoff values of ROS in clinical patients with and without leukocytospermia.

Recently, flow cytometry has gained popularity for semen analysis [18]. Two main advantages of flow cytometry are the small number of spermatozoa it requires and its ability to measure multiple markers simultaneously [19, 20]. These are particularly important features in patients presenting with poor sperm counts. Two specific dyes are available to measure intracellular ROS. Dihydroethidium (DHE) can detect intracellular $O_2^{-\bullet}$ while Dichlorofluorescin (DCFH) can detect intracellular H_2O_2 [21-24].

The goals of this study were to [1] measure basal and stimulated ROS levels by the chemiluminescence method using luminol and lucigenin in different sperm fractions before and after exposure to hydrogen peroxide [2] measure ROS by flow cytometry using DCFH for H_2O_2 and DHE for $O_2^{-\bullet}$ [3] examine the relationship of ROS measurement by the two methods and [4] examine the ability of flow cytometer to detect ROS in samples tested negative by the chemiluminescence method. ROS levels were measured in neat (liquefied seminal ejaculates without any processing), and in mature and immature sperm fractions.

Materials and methods

Sample Collection and Preparation

This study was approved by the Institutional Review Board of the Cleveland Clinic. Semen samples were collected from 18 healthy donors by masturbation after sexual abstinence of at least 48 hours. Of these, three were proven fertile and had fathered a child in the last 2 years and 15 were of unproven fertility. Samples with leukocytospermia (>1 x 10^6 White Blood Cells/mL) were excluded.

A portion of the neat semen samples (liquefied seminal ejaculates without any processing) was separated for performing routine semen analysis as per WHO guidelines [25] and also for the assessment of basal and stimulated ROS levels. To separate mature and immature sperm, liquefied semen was subjected to double density (40% and 80%) gradient centrifugation (PureCeption®, SAGE BioPHARMA, Bedminster, NJ) and centrifuged at 300 g for 20 minutes[26]. The resulting interface between the 40% and 80% layers (immature spermatozoa) was aspirated and resuspended in human tubal fluid media (HTF, Irvine Scientific, Santa Ana, CA). The resulting 80% pellet (mature, highly motile spermatozoa) was aspirated and resuspended in HTF. The resulting 80% pellet (mature, highly motile spermatozoa) was aspirated and resuspended in HTF. In addition, we also examined the effect of sperm concentration (range, 2.5, 5.0, 10.0, and 20 X 10^6 sperm/ mL) on ROS measurement by chemiluminescence using both luminol and lucigenin (Figure 1).

Induction of Artificial OS Measurement of ROS

Basal levels (unstimulated) of ROS were measured in the absence of an exogenous inducer of OS. Stimulated ROS levels were measured by inducing OS in the presence of 50 µL of freshly prepared H_2O_2 (100 µM) to each milliliter of semen specimen and incubating for 15 minutes at 37°C. ROS levels was measured by the conventional chemiluminescence assay using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Chemical Co., St Louis) and lucigenin (bis-*N*-methylacridinium nitrate, Sigma Chemical Co as probes. Test samples consisted of luminol (10 µL, 5 mM) or Lucigenin (4µL, 200 mM) and 400 µL of sperm suspension. Negative controls were prepared by replacing sperm suspension with phosphate buffered saline. Chemilumin-escence was measured for 15 minutes using a Berthold luminometer (Autolumat LB 953; Bad-Wildbad, Germany). Results were expressed as unadjusted ROS X 10^6 counted photon (cpm) or adjusted ROS as X 10^6 cpm/ 20 x 10^6spermatozoa [15].

Determination of reactive oxygen species by flow cytometry

DCFH can detect intracellular H_2O_2 and DHE can detect intracellular $O_2^{-\bullet}$ [19]. DCFH-diacetate (DA, 25 µM) and Hydroethidium (HE) (1.25 µM) obtained from Sigma (Sigma, St. Louis, MO) were added to the sperm suspension and incubated at 25°C for 40

minutes for DCFH-DA and 20 minutes for HE. Each aliquot was analyzed using flow cytometer equipped with 488 nm argon laser as a light source (Becton Dickinson FACScan, San Jose, CA). Green fluorescence (DCFH) was evaluated between 500 and 530nm, while red fluorescence (HE) was evaluated between 590 and 700 nm (excitation: 488 nm; emission: 525 - 625 nm in the FL2 channel). Data were expressed as the percentage of fluorescent spermatozoa. Apoptotic spermatozoa were excluded by using counter stain dye for nucleic acid. We used propidium iodide (PI) as a counter stain dye for DCFH and Yo-Pro-1 as a counter stain dye for the HE (Figure 1).

Flow Cytometry Analysis

All fluorescence signals of labeled spermatozoa were analyzed by the flow cytometer FACScan (Becton Dickinson). A minimum of 10,000 spermatozoa were examined for each assay at a flow rate of <100 cells/sec. The sperm population was gated using 90-degree and forward-angle light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm supplied by an argon laser at 15 mW. PI red fluorescence (580–630 nm) in the FL-2 channel. The percentage of PI positive cells and the mean fluorescence was calculated on a 1023-channel scale and analyzed using the flow cytometer software FlowJo version 6.4.2 (FlowJo, LLC, Ashland, OR)

Statistical Methods

With respect to quantitative measures, comparisons between stimulated and non-stimulated samples within neat, mature, and immature were performed using the Wilcoxon signed-rank test. Pairwise comparisons of neat, mature, and immature within stimulated and non-stimulated samples were also performed using the Wilcoxon signed-rank test. Associations among quantitative variables were measured using Spearman correlation coefficients both within and across sample groups. Linear regression models were used to assess associations, with interaction terms used to test for differences in slopes of relationships for different levels of sample concentration (above and below 5), i.e. effects of concentration on the relationships. Log-transformations (after an addition of 0.1 to avoid undefined values) were applied to skewed variables including Lucigenin, Luminol, DCF, and HE, when performing the regression analyses. Samples classified as positive or negative for adjusted Lucigenin or adjusted Luminol were compared with respect to quantitative sperm quality measures using linear models with generalized estimating equations (GEE) to account for correlation among samples from the same donor. Analyses were performed using R version 2.3.1[27].

Results

The medians (25th, 75th percentile) for neat semen samples were volume 2.9 mL (1.5, 3.9); concentration 42.4 X 10^6/ mL (34.4, 60.4); and percent motility 65.7% (59.3, 70.9). Medians and 25th and 75th percentiles of all other parameters are shown in Table 1.

ROS by chemiluminescence

The ROS levels in unstimulated and stimulated matched (n = 13) as well as unmatched samples (n = 18) showed significant differences in neat, immature, and mature sperm fractions by both luminol and lucigenin as well as by DCF and HE. Neat samples were assessed with HE for superoxide anion.

Nonstimulated (basal; n = 18) ROS Luminol and lucigenin did not show significant differences in basal levels of ROS (either adjusted or nonadjusted) in nonstimulated neat, mature, or immature sperm fractions (Table 1). Immature sperm fractions showed significantly higher levels of basal or adjusted ROS as measured by luminol compared with neat spermatozoa fractions (*P*=.03). A higher, although nonsignificant, level was seen in immature versus mature sperm (*P*=.25). No significant differences were seen in adjusted ROS levels by lucigenin in neat versus mature and immature sperm fractions (*P*=.48 and .67, respectively).

Stimulated ROS (n = 13) significantly higher levels of ROS (both unadjusted and adjusted) as measured by luminol were seen in neat versus mature (*P*=.003 and .009, respectively) and in neat versus immature sperm fractions (*P*=.005 and .003, respectively). No significant differences in levels of ROS (unadjusted and adjusted ROS) with lucigenin were observed between neat versus mature (*P*=.023, and .21, respectively) and between neat versus immature sperm fractions (*P*=.05, and .009, respectively).

Intracellular ROS by Flow Cytometry

ROS levels by flow cytometry in nonstimulated and stimulated neat, immature, and mature sperm by DCH and HE are shown in Figures 2 and 3.

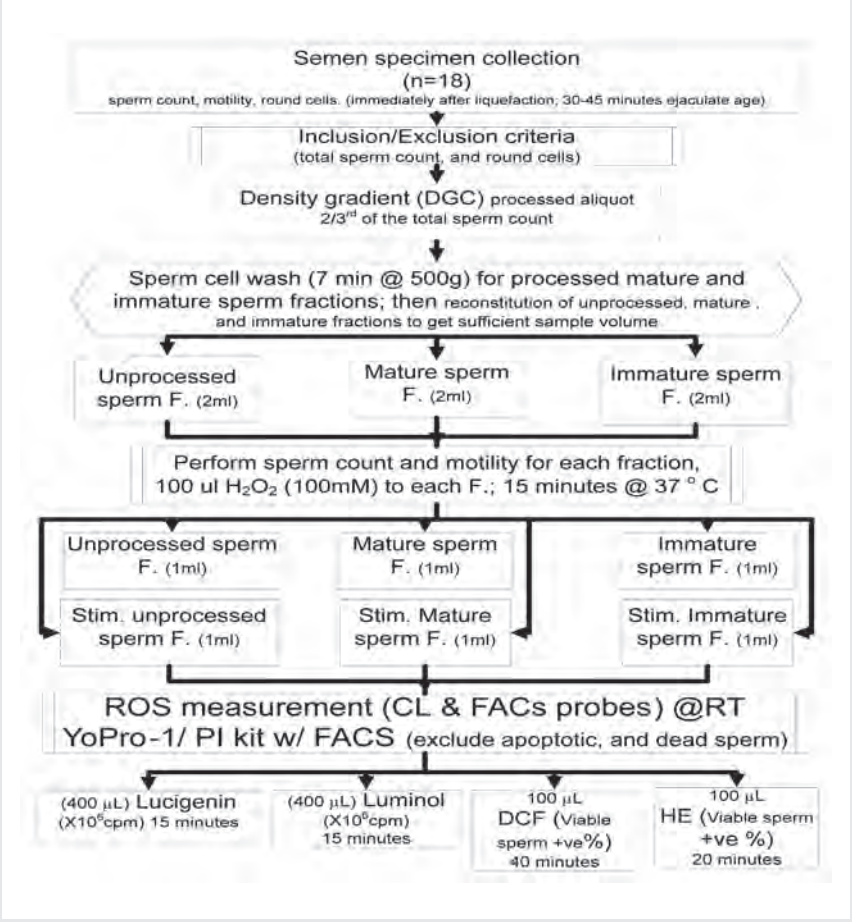
Nonstimulated (Basal; n = 18) ROS The proportions of sperm cells stained for DCF (H_2O_2) and HE ($O_2^{-\cdot}$) were significantly higher in immature sperm fractions compared with mature fractions (*P*=.021 and .003 respectively). Both immature immature and mature sperm fractions had a significantly higher percentage of sperm positive for H_2O_2 compared with neat semen (*P*<.001 and *P*=.003, respectively). On the other hand, the percentage of HE ($O_2^{-\cdot}$)-positive sperm in neat semen was significantly higher compared with mature fractions (*P*<.001) but lower, although nonsignificant, in immature sperm fractions (*P*=.08; Figs. 2 and 3).

Table 1 Comparison of ROS measurement by chemilumescence and flow Cytometry probes in different sperm fractions

Parameter	Neat semen Median (25^{th}, 75^{th})			Mature sperm Median (25^{th}, 75^{th})			Immature sperm Median (25^{th}, 75^{th})		
	Non stimulated (n = 18)	Stimulated (n = 13)	P value	Non stimulated (n = 18)	Stimulated (n = 13)	P value	Non stimulated (n = 18)	Stimulated (n = 13)	p value
Sperm concentration (X 10^6/mL)	14.0 (7.9; 20.7)	9.6 (6.1; 14.1)	0.21	10.2 (7.7; 13.8)	9.3 (3.5; 14.1)	0.72	7.4 (3.2; 9.3)[b]	3.5 (2.5; 5.4)[bb]	0.21
DCFDH +ve sperm (%);(H_2O_2)	3.2 (1.6; 9)	42.2 (35.7; 46.6)	<0.001	20.4 (5.7; 36.1)[a]	57.0 (32.4; 65.4)	0.003	32.5(14.4; 41.8)[b]	52.1(30.9; 61.7)	0.031
ROS (luminol) (X 10^6 cpm)	0.1 (0; 0.2)	0.4 (0.1;5.7)	0.028	0.1 (0; 0.2)	58.3 (40.7; 74.8)[aa]	<0.001	0.1 (0; 0.2)	49.5 (34.4;68.1)[bb]	<0.001
Adjusted ROS (luminol) (X 10^6 cpm/20 X 10^6 sperm)	0.1 (0; 0.2)	1.3 (0.1; 6.8)	0.037	0.2 (0; 0.7)	111.8 (61.5; 415.4)[aa]	<0.001	0.2 (0.1; 1.8)	165.7 (115.5; 412.1)[bb]	0.001
DHE +ve sperm (%);($O_2^{-\bullet}$)	3.2 (1.5; 4.4)	7.2 (2.0; 10.3)	0.06	0.9 (0.6; 1.9)[a]	13.3 (3.0; 23.2)	0.001	2.4 (1.5; 3.0)[c]	5.4 (2.7; 16.3)	0.012
ROS (lucigenin) (X 10^6 cpm)	0.0 (0; 0.2)	0.7 (0.4; 1.9)	0.001	0.0 (0; 0)[a]	3.0 (1.1; 4.6)	<0.001	0.0 (0; 0)[b]	3.5 (1.2; 5.3)	<0.001
Adjusted ROS (lucigenin) (X 10^6 cpm/20 X 10^6 sperm)	0.0 (0; 0.2)	2.0 (0.7; 7.1)	0.001	0.0 (0; 0)	8.2 (3.3; 14.5)	<0.001	0.0 (0; 0)	11.2 (4.4; 25.9)	<0.001

SD = Standard deviation; IQR = interquartile range; a = significant difference between mature and neat sperm fractions; b = significant difference between immature and neat sperm fractions; c = significant difference between mature and immature sperm fractions; aa = significant difference between stimulated mature and stimulated neat sperm fractions; bb = significant difference between stimulated immature and stimulated neat sperm fractions; P<0.05 considered significant using p

Figure 1 A representative flowchart for this study experimental steps and the chronologic methodology.



Stimulated ROS (n = 13) Both H_2O_2 (DCF^{+ve}) and $O_2^{-\cdot}$ (HE^{+ve}) levels were increased in neat (P<.001 and .021, respectively), mature (P=.009 and .002, respectively), and immature (P=.24 and .002, respectively) sperm fractions. Also, mature sperm fraction showed higher poststimulation levels of both H_2O_2 (DCF^{+ve}) and $O_2^{-\cdot}$ (HE^{+ve}) compared with the neat (P=.14 and .2, respectively) and immature (P=.69 and .26, respectively) sperm fractions (Figs. 2 and 3).

Figure 2 Show comparison of semen specimen in different processing and treatment regarding; **A**: level of intracellular H_2O_2 (DCF +ve) spermatozoa percentage, **B**. level of intracellular $O_2^{-\cdot}$ (HE+ve sperm percentage)

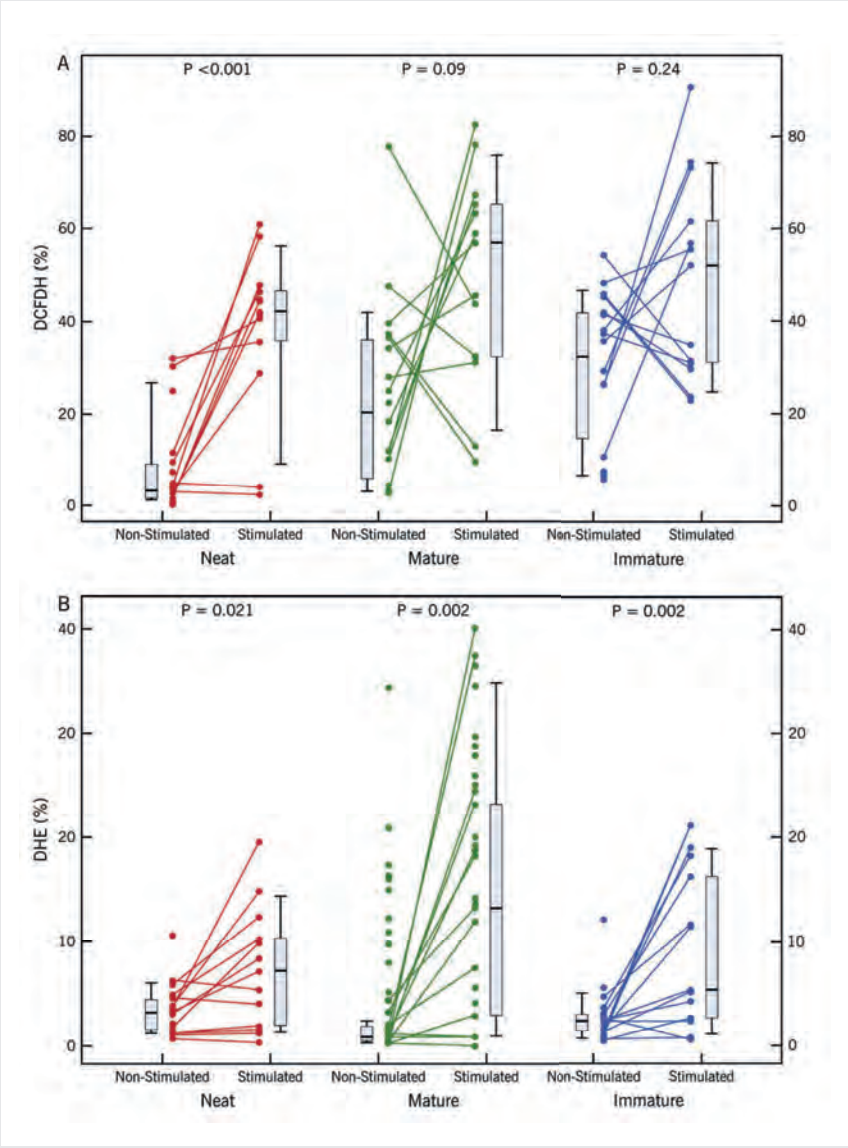


Table 2 Genotype and Allele frequencies for the eNOS gene single nucleotide polymorphism (Glu298Asp variant) in asthenozoospermic patients and healthy controls

Parameters	DCF $^{+ve}$ sperm (%)			HE $^{+ve}$ sperm (%)		
	r	p-value	Effect of conc.§ P value	r	p-value	Effect of conc. § P value
Luminol (X 10^6 cpm)	0.57	<0.001	0.37	0.402	<0.001	0.86
Adjusted Luminol (X 10^6 cpm/ 20 million sperm)	0.621	<0.001	0.1	0.32	0.003	0.7
Lucigenin (X 10^6 cpm)	0.51	<0.001	0.46	0.573	<0.001	0.36
Adjusted Lucigenin (X 10^6 cpm/ 20 million sperm)	0.56	<0.001	0.08	0.49	<0.001	0.54
DCF^{+ve} sperm (%)	----	-----		0.38	<0.001	0.31
HE^{+ve} sperm (%)	0.38	<0.001	0.35			

§ log values were used.

Correlation of ROS by Flow Cytometry and Chemiluminescence

The percentage of DCF^{+ve} sperm showed significant correlation with unadjusted luminol (r = 0.57, *P*<.001), adjusted luminol (r = 0.62, *P*<.001), unadjusted lucigenin (r = 0.51, *P*<.001), and adjusted lucigenin (r = 0.56, *P*<.001). The percentage of HE^{+ve} sperm had significant correlations with unadjusted luminol (r = 0.40, *P*<.001), adjusted luminol (r = 0.32, P=.003), unadjusted lucigenin (r = 0.57, *P*<.001), and adjusted lucigenin (r = 0.49, *P*<.001; Table 2).

Adjusted ROS values by chemiluminescence increase the correlation with the intracellular H_2O_2 level as measured by DCF. However, this correlation with the intracellular $O_2^{\cdot}$ by HE decreases with adjusted ROS values in the chemiluminescence results for the same specimen. DCF^{+ve} sperm is also correlated with percentage of HE^{+ve} sperm in a given sample (r = 0.38, *P*<.001).

When we examined the effect of low and high sperm concentration after log transformation, regression analysis showed no significant effect of concentration on correlation of ROS by flow cytometry and chemiluminescence (Table 2).

Evaluation of the Sperm Concentration on Chemiluminescence Results

No effect of sperm concentration (2.5–20 x 10^6 sperm/mL) was seen in ROS levels by luminol (unadjusted *P*=.15; or adjusted *P*=.07) or lucigenin (*P*=.53). The Spearman correlation showed significant negative dependence of the adjusted (r = 0.887, *P*<.001) or log-adjusted lucigenin (r = 0.887, *P*<.001) on sperm concentration.

Basal Intracellular ROS Level Measured by Flow Cytometry

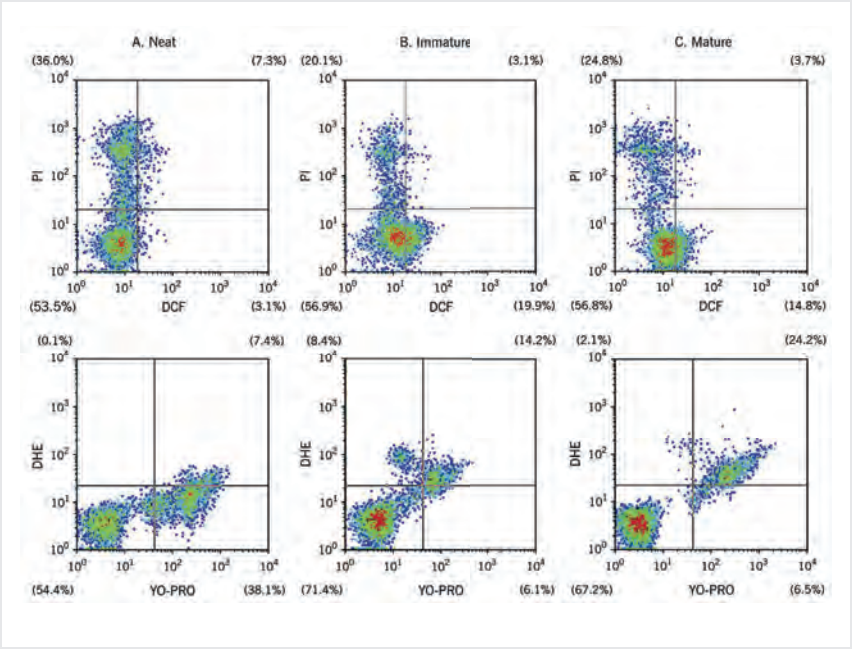
The basal levels of H_2O_2 and $O_2^{\cdot}$ in neat semen of both proven and unproven donors (n = 18) were higher (7.9 ±10.3 and 3.4 ± 2.5). Proven fertile men (n = 3) showed lower basal ROS levels in the neat samples with both DCF (5.0 ± 4.1) and HE (3.4 ± 1.6).

Comparison of ROS negative samples by flowcytometry

When all aliquots (stimulated and unstimulated) were analyzed, 13 aliquots tested negative by luminol (ROS ≥ 0 cpm/20 X 10^6 sperm), and 74 tested positive by luminol (ROS ≥ 0.2 cpm/20 X 10^6 sperm; cutoff established by our lab). In contrast, both DCF and HE were able to detect ROS in all luminol-negative samples. Positive luminol samples showed significantly higher levels by DCF (33.9 ± 2.7; *P* =.0002) and HE (6.58 ± 0.98; *P* =.006) compared with luminol-negative aliquots (14.5 ± 4.6 for DCF and 3.02 ± 0.80 for HE-positive samples; Fig. 4).

Similarly, when we compared 44 lucigenin-negative and 43 lucigenin-positive aliquots by flow cytometry, both DCF and HE were able to detect ROS in lucigenin-negative samples. Lucigenin-positive aliquots showed significantly higher levels by DCF (41.7 ± 3.8; P<.0001) and HE (9.57 ± 1.50; *P* < .0001) compared with aliquots that tested negative by lucignin (20.6 ± 2.5 for DCF and 2.6 ± .42 for HE-positive samples; Fig.4)

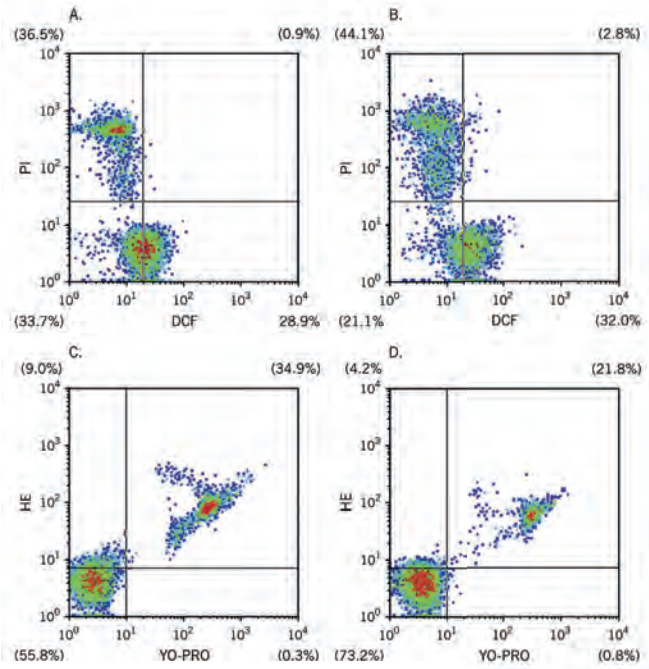
Figure 3 Dotplot histograms showing the simultaneous measurements of **Upper:** intracellular hydrogen peroxide and intracellular superoxide in **A.** neat, **B.** immature and **C.** mature spermatozoa. For **hydrogen peroxide (Upper):** Lower left quadrant represents viable, non-stained sperm, while lower right shows viable sperm with high intracellular H_2O_2. Upper left quadrant shows apoptotic sperm. Upper right shows apoptotic sperm with high intracellular H_2O_2. For **Superoxide (Lower):** Lower left quadrant represents viable, nonstained sperm, while lower right shows apoptotic sperm. Upper left quadrant shows viable sperm with high intracellular $O_2^{-\cdot}$ and upper right shows apoptotic sperm with high intracellular $O_2^{-\cdot}$. The numbers in parenthesis represent the sperm population in each quadrant



Discussion

In our study, we validated that flow cytometry is as useful and accurate as the chemiluminescence assay for the measurement of ROS in human semen. Furthermore, by using both DCF and HE probes, we can simultaneously measure the two main types of ROS generated intra-cellularly, namely, H_2O_2 and $O_2^{-\cdot}$. The increase in ROS values detected by both DCF and HE probes after exposure to H_2O_2 confirms their ability to specifically measure H_2O_2 and $O_2^{-\cdot}$. This is in agreement with Guthrie and Welch [19], who reported that these probes can selectively measure H_2O_2 and $O_2^{-\cdot}$. The significant relationships between DCF and luminol (H_2O_2) and HE and lucigenin ($O_2^{-\cdot}$) additionally

Figure 4 Flow cytometry histograms showing the intracellular ROS measured by DCH for H_2O_2 could be detected in chemiluminescence negative specimen; **A and B**: luminol negative and positive samples **C-D:** lucigenin negative and positive samples.



validate these probes for sperm ROS measurements. Another important finding that we are reporting for the first time is that intracellular H_2O_2 can be detected using flow cytometry even in aliquots that test negative by chemiluminescence.

Flow cytometry is highly reproducible technology that allows simultaneous measurement of multiple parameters in a selected cell population. Recently, it was shown to accurately measure sperm count and other parameters such as anti-sperm antibodies as well as sperm DNA damage [28]. Similar data have been reported on the assessment of sperm concentration, apoptosis, and leukocyte concentration using a multi-parameter flow cytometry for a simultaneous rapid and accurate evaluation of sperm parameters [20]. In addition, other investigators described the flow cytometry method for measuring intracellular ROS in semen samples from animals using both DCF and HE stains [19, 21, 29] or HE only [30]. De Iuliis validated the use of HE as a probe together with flow cytometry to measure OS and compared this with other methods

such as high pressure liquid chromatography (HPLC) and chemiluminescence for the investigation of intracellular $O_2^{-\cdot}$ production [30]. While there are reports on normal ROS levels detected by chemiluminescence in fertile men, similar data on intracellular ROS using HE as a probe together with flow cytometry are lacking [17].

In our study, we demonstrated that specific intracellular ROS ($O_2^{-\cdot}$ and H_2O_2) levels can be measured simultaneously in human spermatozoa with flow cytometry using DCF and HE probes. Our results showed that neat, mature, and immature spermatozoa had different levels of intracellular ROS. Oxidative sperm damage occurs when the intracellular ROS levels exceed the available antioxidant capacity of the cells. OS may cause damage to the sperm DNA, proteins, and lipids, leading to male factor infertility [31-33]. ROS is produced both by leukocytes and abnormal spermatozoa [30, 34, 35]. Therefore, measuring the intracellular ROS is valuable in determining the source of excess ROS compared with measuring global intra- and extracellular ROS using chemiluminescence.

We are reporting for the first time the basal levels of the intracellular ROS for neat, immature, and mature sperm fractions. We observed a shift in the pattern of $O_2^{-\cdot}$ and H_2O_2 levels in neat semen and in immature and mature sperm fractions prepared by double-density gradient centrifugation. This has not been reported earlier in the literature as largely global (intra- and extracellular) ROS levels were measured. Taking into account the advantages of flow cytometry, this might be a useful method for accurate studies on ROS in infertile men. In addition, it will provide more information on the specific ROS levels selectively in viable spermatozoa.

Measuring ROS levels using flow cytometry has many advantages such as [1] speed, [2] accuracy, and [3] reproducibility. In addition, our study demonstrated that flow Cytometry detected intracellular ROS levels even in samples that tested negative by chemiluminescence. The clinical implication of our finding is that samples that would otherwise have tested negative for ROS chemiluminescence may still be compromised, with sperm damage resulting in impaired fertilization potential.

Another significant advantage of using flow cytometry to measure ROS levels is that semen samples with very low sperm count (oligozoospermia) can be accurately evaluated. There is evidence that sperm count is declining [36], and more men with very low sperm counts (with sperm concentration $<5 \times 10^6$/ mL) are opting for assisted reproductive technologies such as IVF/intracytoplasmic sperm injection [36, 37]. Flow cytometry provides a reliable means of assessing ROS production at such low concentrations

We previously reported that chemiluminescence may be accurate and reliable but only in samples with sperm concentration >1 million/mL [12, 15]. Its sensitivity declines significantly even in specimen with sperm concentrations <5 million/mL [14, 15]. In this current study, we performed measurements by luminometer using sperm samples with low concentration and found similar values for specimen with sperm concentrations of 2.5 and $>5 \times 10^6$/mL.

Our findings showed that using adjusted ROS values improves the relationships of lucigenin and luminol with the intracellular H_2O_2 level, but it may weaken such a relationship with the intracellular $O_2^{-\cdot}$ level. This shows that adjusting sperm concentration is critical for intracellular H_2O_2, which is the main free radical detected by the luminometer [38]. One of our study limitations was the relatively small sample size. We are planning a large-scale study using both fertile and infertile specimens, including those from oligozoospermic men, to compare both technologies in terms of sensitivity and specificity and to identify the best cutoff value for each probe.

In conclusion, we have standardized ROS measurement using the flow cytometry specific probes for simultaneous detection of both intracellular $O_2^{-\cdot}$ and H_2O_2 selectively in viable human spermatozoa. We are reporting for the first time the basal levels of the intracellular ROS for neat, immature, and mature sperm fractions. Flow cytometry and chemiluminescence may be comparable in specimens producing high levels of ROS; however, flow cytometry is superior in samples generating low levels of ROS and in low-concentration (oligospermic) semen specimens. Use of adjusted ROS levels is useful in ROS measurement by chemiluminescence. Luminol and lucigenin results are related to intracellular H_2O_2 and $O_2^{-\cdot}$ levels, confirming their global affinity to any free radical. Specimen testing negative by chemiluminescence assay may still have the ability to produce intracellular H_2O_2, which can be easily detected by flow cytometry.

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Chapter 8

Semen characteristics and sperm DNA fragmentation in infertile men with low and high levels of seminal reactive oxygen species

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Abstract

Objective: Little is known about the clinical and laboratory findings in high reactive oxygen species (ROS) conditions. Our aim was to examine sperm motility, total antioxidant level (TAC), DNA fragmentation and medical history in infertile men with high seminal ROS.
Design: Prospective study**.**
Settings: Tertiary care hospital**.**
Patients: Infertile men (n=101)
Interventions: Group I (n = 57) included men with seminal ROS (<250 RLU/sec/X 10^6 sperm) while group II (n = 44) included men with seminal ROS levels (≥250 RLU/sec/X 10^6 sperm).
Main Outcome Measures: Seminal ROS, TAC, sperm DNA fragmentation**,** ROS/TAC score were measured.
Results: Group II had high incidence of sperm DNA fragmentation than group I (p = 0.036). The odds ratio of 1.25 for elevated ROS levels corresponded to >10% greater DNA fragmentation in our patients (95%CI 1.01 - 1.53). Group II showed poor motility (p = 0.001), a higher incidence of leukocytospermia (p = 0.001) and higher ROS-TAC scores (p<0.001) compared with group I. ROS was negatively correlated with sperm curvilinear velocity (VCL) (r = -0.24, p = 0.021), linearity (r = -0.24, p = 0.017) and sperm motility (r = -0.31, p = 0.002). Sperm motility was correlated with %TUNEL^{+ve} sperm (r = -0.39, p<0.001).
Conclusions: An increase in seminal ROS levels by 25% was associated with a 10% increase in sperm DNA fragmentation. Sperm motility was affected by seminal ROS and sperm DNA fragmentation.

Keywords: sperm motility, sperm motion kinetics, male infertility, sperm DNA fragmentation, oxidative stress, reactive oxygen species, total antioxidant capacity

Introduction

Basal levels of ROS are essential for physiological sperm functions [1, 2]. However, high seminal ROS levels lead to oxidative stress (OS), which in turn can reduce sperm motility, viability and fertilizing potential and increase sperm DNA fragmentation rates [3-7]. Some authors report high ROS levels to be an independent marker for male factor infertility [3].

Introducing seminal ROS assay in clinical andrology laboratory services may help clinicians in accurate diagnosis of their patients such as idiopathic cases or other diagnostic conditions associated with oxidative stress induced sperm damage [6, 8-10]. New OS scores (such as ROS-TAC score) have been added to easily diagnose infertile patients with OS, however seminal ROS still remains the main parameter for measuring OS [11]. High levels of seminal ROS may be an important indicator of further deterioration of sperm quality in men with OS.

In a preliminary study (unpublished data) we observed all seminal ROS values in the healthy donors (proven and unproven healthy) were < 250 relative light units (RLU)/ second$X10^6$ sperm. We therefore took this cutoff to differentiate infertile men with values > 250 RLU/sec/ $X10^6$ sperm and categorize these patients with high seminal ROS that are vulnerable to OS induced sperm damage. The objective was to compare infertile men with normal and high levels of ROS in seminal ejaculates (as determined from unpublished data from our group) and examine their sperm parameters and clinical information.

Materials and methods

Subjects Selection

This prospective study was approved by the Institutional Review Board of our hospital. Our patient population consisted of 101 infertile patients who attended our fertility clinic between 2008 and 2009 for male factor fertility. All patients underwent semen analysis according to World Health Organization guidelines [12].

Semen Collection and Preparation

Semen specimens were collected and analyzed as per WHO 1999 guidelines. Briefly, 5 µL of liquefied specimen was loaded on a 20 µL Cell-Vu® chamber (Millennium Sciences, Inc., New York, NY). Leukocytospermia was confirmed (using the Endtz test) in any specimen showing round cells > 0.2 $X10^6$ white blood cells/mL of semen [13, 14]. Sperm motion characteristics such as linearity (%) and curvilinear velocity (VCL, µm/ sec) were examined by computer assisted sperm analysis (CASA, IVOS version 10, Hamilton Thorne, Inc., Beverly, MA) [15].

The remaining seminal ejaculate was divided into 3 aliquots for further use. One aliquot was used to measure seminal ROS levels. The second aliquot was used to assess DNA damage via the TUNEL (terminal dUTP nick-end labeling) assay. Spermatozoa were fixed and kept in 70% ethanol at -20°C for the TUNEL assay. The third aliquot was centrifuged at 1,000 X g for 7 minutes, and clear seminal plasma was aliquoted and stored at -70°C for the measurement of the total antioxidant capacity (TAC).

Measurement of reactive oxygen species (ROS)

Seminal ejaculates that had not undergone any additional processing (neat samples) were used for ROS measurement by chemiluminescence assay. Luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Chemical Co., St Louis, MO) was used as a probe. A 100 mmoL/ L stock solution of luminol was prepared in dimethyl sulfoxide. For the analysis, 10 µL of the working solution (5 mM) was added to 400 µL of a neat sperm sample [16, 17]. Chemiluminescence was measured for 15 minutes using a luminometer (Autolumat LB 953; Berthold Technologies, LLC, Oak Ridge, TN). The results were expressed as RLU/ sec/10^6 sperm.

Patients were divided into 2 groups based on their ROS levels. Group 1 consisted of patients with normal levels of seminal ROS (< 250 RLU/sec/ X10^6 sperm; n = 57), which was used as a control group, and group 2 consisted of patients with high levels of seminal ROS (> 250 RLU/sec/ X10^6 sperm; n = 44). This cutoff point was derived from an unpublished study performed by our group that showed all seminal ROS values in healthy donors (proven and unproven healthy) were < 250 relative light units (RLU)/ secondX10^6 sperm.

Total antioxidant (TAC) assay

The TAC of the seminal plasma samples was measured using an antioxidant assay kit (Cayman Chemical Company, Ann Arbor, Michigan). Its principle is based on the ability of aqueous- and lipid-based antioxidants in seminal plasma to inhibit oxidation of the ABTS® (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS®$^{\cdot+}$. Under the reaction conditions used, the antioxidants in the seminal plasma suppress absorbance at 750 nm to a degree that is proportional to their concentration. The capacity of the antioxidants in the sample to prevent ABTS® oxidation was compared with that of Trolox, a water-soluble tocopherol analogue, and the results were reported as micromolar trolox equivalents [18].

Calculation of ROS-TAC score

The principal component (PC) was used to calculate the ROS-TAC score for all patients. This was derived from their seminal ROS and seminal plasma TAC values [19] with some modifications.

ROS-TAC = 50 + (10/ 0.99672)*[-0.707* StdLogROS + 0.707* StdTAC]

Where

LogROS = Log10 (ROSRLU + 5)
StdLogROS = Standardized Log ROS = (LogROS - 2.3814)/(1.2357)
StdTAC = Standardized TAC = (TAC - 1976.56)/ (641.68)

Sperm DNA fragmentation

Sperm DNA fragmentation was evaluated with the terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL) assay using Apo-Direct™ kit (Pharmingen, San Diego, CA) as established earlier [20, 21]. Briefly, 1 to 2 million spermatozoa were washed in phosphate buffered saline (PBS), resuspended in 3.7 % paraformaldehyde with adjustment of the concentration to 1-2 X 10^6 sperm/ mL and placed on ice for 30 to 60 minutes at 4°C. Thereafter, spermatozoa were again washed to remove the paraformaldehyde and then re-suspended in 70% ice-cold ethanol. Specimens were kept at -20°C until the run time. In addition we also included an internal set of samples which were tested negative or positive for DNA damage with each run. Following a second wash in PBS to remove the ethanol, sperm pellets were re-suspended in 50 µL of the freshly prepared staining solution for 60 minutes at 37°C. According to the manufacturer's instructions, the staining solution contains terminal deoxytransferase (TdT) enzyme, TdT reaction buffer, fluorescein isothiocynate tagged deoxyuridine triphosphate nucleotides (FITC-dUTP) and distilled water. All specimens were further washed in rinse buffer to remove the unbound reaction solution, re-suspended in 0.5 mL of propidium iodide/RNase solution and incubated for 30 minutes in the dark at room temperature. Flow cytometric analysis was then performed. The percentage of positive cells ($TUNEL^{+ve}$) was calculated on a 1023-channel scale using the flow cytometer software (FlowJo Mac version 8.2.4, FlowJo, LLC, Ashland, OR) [22].

Review of medical charts and related parameters

A complete medical history and clinical examination were performed for every patient. Their medical charts were reviewed for age, body mass index (BMI), primary or secondary infertility and duration of infertility. A history of antioxidant and/or antibiotic prescription use was also verified.

Statistical Analysis

The differences in distributions of OS-induced damage markers and patient characteristics between patients with ROS levels below vs. above 250 RLU/ sec/10^6 sperm were assessed using Wilcoxon rank sum and Chi-square tests. Summaries of the distributions–mean, standard deviation, median, and interquartile ranges (IQR)—were examined. Spearman correlations were used to assess associations between ROS levels and other measured parameters. Statistical significance was considered when $p < 0.05$. All analyses were performed using R version 2.3.1 (www.r-project.org) [23].

Results

OS-induced sperm damage markers

There were no significant differences between group I (normal seminal ROS levels) and. group II (high seminal ROS levels) regarding seminal plasma TAC levels (p = 0.43). However, group I had a significantly higher ROS-TAC score (p<0.001) than group II.

Table 1 Clinical and semen parameters in infertile men with high or physiological seminal ROS values

Variable	Group I (ROS<250) (n=57) (56.4%)	Group II (ROS≥250) (n=44) (43.6%)	P-value
Age (years)	36.53 ± 7.07	37.33 ± 8.25	0.77
Height (cm)	177.69 ± 8.73	179.08 ± 9.70	0.49
Body weight (kg)	90.98 ± 17.43	92.46 ± 16.79	0.44
Body mass index (BMI)	28.81 ± 4.96	28.42 ± 3.94	0.99
Infertility Type:			0.51
Primary	33 (61.1%)	24 (54.5%)	
Secondary	21 (38.9%)	20 (45.5%)	
Infertility duration (years)	2.18 ± 2.04	2.38 ± 2.28	0.68
Abstinence time (days)	3.45 ± 1.33	3.79 ± 1.68	0.32
Semen pH	7.63 ± 0.25	7.65 ± 0.22	0.71
Ejaculate volume (mL)	2.88 ± 1.45	3.26 ± 1.68	0.35
Sperm Motility (%)	62.56 ± 22.87	45.21 ± 28.**22**	0.001
Curvilinear velocity (VCL) µm/sec	42.00 ± 8.21	37.76 ± 12.28	0.005
Linearity (%)	49.45 ± 9.24	46.15 ± 9.77	0.026
WHO normal Morphology (%)	15.33 ± 10.69	14.30 ± 10.99	0.63
Strict criteria normal sperm (%)	2.82 ± 3.24	2.24 ± 2.54	0.41
Total antioxidant capacity (TAC) (µmol trolox equivalent/ml)	2022.81 ± 696.68	1916.78 ± 565.55	0.43
ROS-TAC score	53.96 ± 8.46	26.05 ± 10.02	<0.001
Sperm TUNEL^{+ve} %	22.97 ± 19.12	33.83 ± 25.80	0.055

Results are presented as mean ± standard deviation (SD), frequency (%). Associations with categorical variables were assessed by chi-square tests. Associations with quantitative and ordinal variables were assessed by Wilcoxon rank sum tests.

There was a higher incidence of sperm DNA fragmentation expressed as %TUNEL^{+ve} in group II (p = 0.036) (Table 1). The odds ratio of elevated ROS corresponding to a 10% greater DNA fragmentation was estimated to be 1.25 (95% CI 1.01 to 1.53).

Semen parameters

Abstinence time (p = 0.32), ejaculate volume (p = 0.35), semen pH (p = 0.71), and normal sperm morphology by WHO (p = 0.63) and strict Kruger criteria (p = 0.41) were comparable between the 2 patient groups.

The percentage of motile sperm (p = 0.001), VCL µm/sec (p = 0.005), and linearity (p = 0.026) were all lower in group II (Table 1). The incidence of leukocytospermia was higher (73.9%) in group II (Table 2) vs. group I (26.1%; p = 0.004).

Table 2 Incidence of leukocytospermia with antibiotic and antioxidant prescription within infertile patient groups

Variable	Group I (ROS<250) (n=57) (56.4%)	Group II (ROS≥250) (n=44) (43.6%)	P-value
Leukocytospermia (ENDTZ test) (x10^6/ml	0.05 ± 0.15	0.64 ± 1.39	0.001
Antibiotic prescription			0.10
No	46 (80.7 %)	29 (65.9 %)	
Yes	11 (19.3 %)	15 (34.1 %)	
Antioxidant supplementation			0.041
No	40 (71.4%)	22 (51.2%)	
Yes	16 (28.6%)	21 (48.8%)	

Results are presented as mean ± standard deviation (SD), frequency (%). Associations with categorical variables were assessed by chi-square tests. Associations with quantitative and ordinal variables were assessed by Wilcoxon rank sum tests.

Patient-related parameters

There were no significant differences between infertile patient groups with regards to patient age, height, weight, or BMI. No significant differences were observed in infertile men in the two groups in regards to duration or type of infertility (Table 1).

Antibiotics were prescribed in 11 (19.3%) patients in group I vs. 15 (34.1%) of patients group II (p = 0.1) Antibiotics were recommended for a comparable duration (1 to 3 months) in both patient groups (p = 0.7). Antioxidant supplementation was recommended

for 16 (28.6%) patients in group I vs. 21 (48.8%) patients in group II (p = 0.04). Antioxidant supplementation were recommended for a longer duration (up to 4 months) in group II vs. group I (p = 0.03) (Table 2).

Correlation studies

As a secondary analysis, spearman correlation showed positive correlations between seminal ROS levels and the Endtz test (r = 0.41, p<0.001) and with sperm DNA fragmentation (%TUNEL^{+ve}) (r = 0.21, p = 0.06). ROS levels were negatively correlated with VCL (r = -0.24, p = 0.021), linearity (r = -0.24, p = 0.017) and sperm motility (r = -0.31, p = 0.002). Sperm motility showed negative correlation with %TUNEL^{+ve} (r = -0.39, p<0.001; Figure 1). Negative correlations were observed between seminal

Figure 1 Plots showing ROS relationship with **A**: sperm curvilinear velocity (VCL) **B**: linearity **C**: total motile sperm and **D**: percentage of TUNEL^{+ve} sperm.

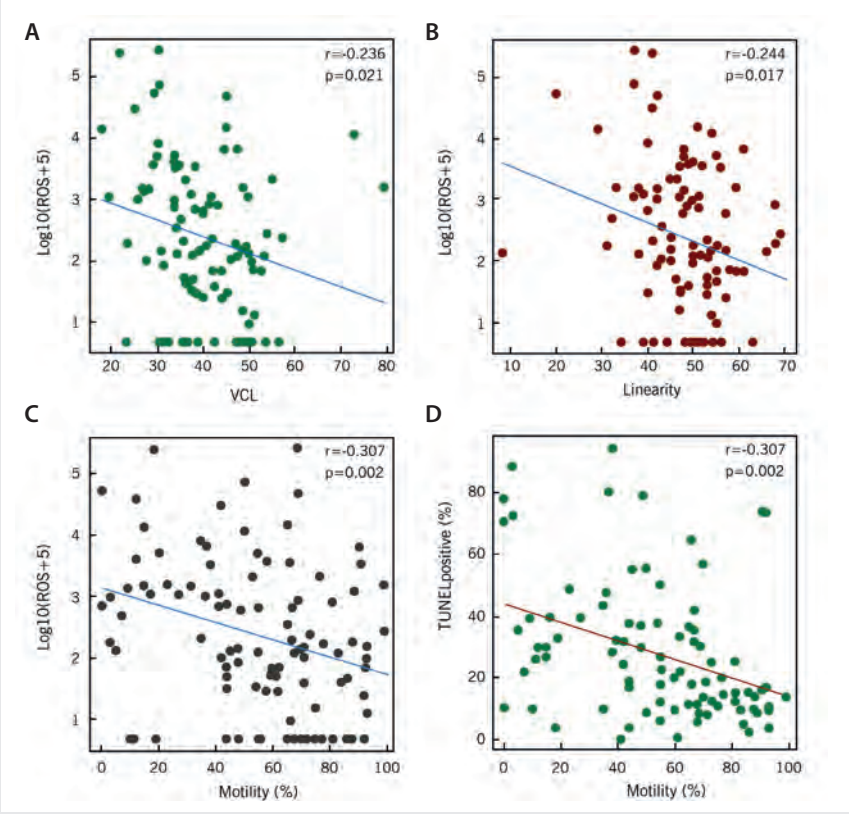
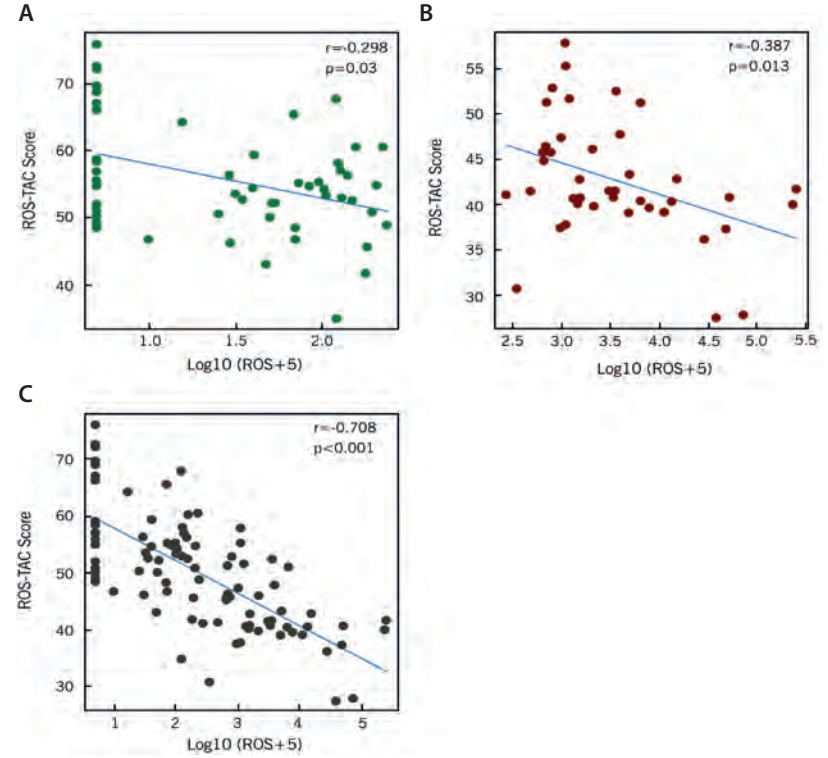


Figure 2 Plots showing the relationship of ROS-TAC score with **A**: ROS levels in overall patients group **B**: seminal ROS levels in patients with high ROS >RLU/ sec/10^6 sperm (group 1) and **C**: patients with low ROS <250 RLU/ sec/10^6 sperm (group 1).



ROS values with ROS-TAC score (r = -0.71, p<0.001). The ROS-TAC score was negatively correlated with levels of ROS in all patients (r = -0.7, p<0.001), group 1 patients with seminal ROS> 250 (r = -0.39, p = 0.013), and in group 2 patients with seminal ROS <250 (r = -0.30, p = 0.03) (Figure 2).

Discussion

Our understanding of semen characteristics and patient characteristics associated with high seminal ROS generation is poor. Therefore, our study aimed to compare sperm DNA fragmentation, OS markers, and other routine infertility parameters in

men with normal vs. high seminal ROS generation. In this study, we only enrolled infertile patients who were seeking medical help for male factor related fertility issues. The patients' ages, infertility type/duration, and BMI had no effect on seminal ROS values. The two patient groups were comparable in terms of abstinence, semen pH, ejaculate volume, percentage of normal morphologically spermatozoa, and seminal TAC. These findings agree with previous findings suggesting that routine sperm parameters overlap between infertile and fertile men [24], indicating that seminal ROS is an independent parameter for male infertility [3]. The patient groups had comparable levels of seminal plasma TAC. However, the ROS-TAC score demonstrated OS conditions in the infertile men with high seminal ROS levels. This agrees with our earlier reports recommending seminal ROS measurement as a routine test for infertile men [25] and the use of the ROS-TAC score over the use of just ROS or TAC o in these patients [19, 26].

Infertile men with high seminal ROS levels had a lower percentage of motile sperm, VCL, and linearity [27]. This can be explained by the disturbance of the energy supply that may occur following a disturbed mitochondrial membrane potential, which in turn causes high levels of ROS to be released into the cytoplasm and depletion of the energy supply that affects both sperm motility and kinetics.

Our study provides evidence for OS-induced sperm DNA damage in infertile men with high seminal ROS levels. Infertile men may have high sperm DNA fragmentation that can be explained by the presence of additional risks factors with OS-associated conditions such as varicocele or other lifestyle issues as reported earlier [1, 28-30].

Although several recent reports have attempted to define levels of sperm DNA fragmentation in infertile men, additional studies are needed before the tests that measure this parameter can be recommended for routine clinical use in infertile men [31, 32]. Seminal plasma TAC was comparable in both groups of our study, despite the difference in seminal ROS levels. This may be related to whether or not multivitamin/ antioxidants supplements were prescribed. Furthermore these findings also indicate that seminal TAC may vary during this compensatory phase in an attempt to scavenge the increasing levels of ROS. In an earlier study, we reported TAC cutoff values, in this study all patients had seminal plasma TAC levels within normal reference range [18]. This finding confirms the above assumption which indicates infertile men with TAC levels below 1420 µM trolox equivalent/ mL may have depleted antioxidant capacity and are therefore more vulnerable to OS-induced damage. With seminal TAC below 1420 µM trolox equivalent/ mL, OS-induced sperm damage may occur even with low seminal ROS values. This again points to the importance of calculating the ROS-TAC score for these patients as it is more reflective of the actual OS status. Furthermore, our current study findings also point to the need to develop new OS markers and scores (such as ROS-TAC) that are more sensitive and account for more than one parameter of OS status. This also is in agreement with a previous report [8].

The high incidence of leukocytospermia in the infertile men with high seminal ROS levels may represent an additional cause of low sperm quality. Antibiotic supplementation may not be effective in normalizing ROS generation or protect against further deterioration in sperm quality. Doxycycline has been reported to induce sperm damage with in vitro treatment [33, 34]. Moreover, the negative relationships of seminal ROS with sperm motility and its kinetics in all patients confirm that sperm damage occurred as a result of high ROS generation in these infertile men.

Our study showed a positive relationship between seminal ROS and leukocytospermia and sperm DNA fragmentation. We recommend examining infertile patients for leukocytospermia and for sperm DNA integrity in order to improve their clinical management.

Because our study was limited to diagnose the presence or absence of ROS, we recommend follow up studies to develop new antibiotic/antioxidant strategies for these patients. Also, we did not further classify our patients based on their clinical diagnoses. We recommend more studies on specific clinical diagnosis and specific age groups to identify the seminal plasma reference values in those specific groups.

In conclusion, infertile men with high seminal ROS levels have high incidence of sperm DNA fragmentation. An increase of seminal ROS by 25% may be associated with a 10% increase in sperm DNA fragmentation. The percentage of total motile spermatozoa was negatively related to seminal ROS and sperm DNA fragmentation. The ROS-TAC score was better than ROS or TAC values alone thereby differentiating infertile men with OS from those without OS. Novel OS markers should be developed to help better identify infertile men with DNA damage. Newer strategies are required for male infertility oxidative stress management. We recommend establishing reference values for diagnostic/ prognostic use of oxidative stress markers in andrology that may lead to more optimal treatments for infertile men with OS-induced sperm damage.

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Chapter 9

Reactive oxygen species levels are independent of sperm concentration, motility and abstinence in a normal, healthy proven fertile man - a longitudinal study

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Abstract

In a longitudinal study over a period of 21 months, we demonstrated that seminal ROS levels are independent of sperm concentration, motility or abstinence duration within a healthy sperm donor, although some variations were observed in ROS levels. We suggest that fluctuation in seminal ROS values may be related to physiological or transient changes in spermatogenesis.

Key words: Semen analysis; Reactive oxygen species; variability; Male infertility**.**

Capsule:
ROS levels are independent of sperm concentration, motility and abstinence period in normal, healthy proven fertile man. At physiological level ROS value do not affect sperm parameters.

Reactive oxygen species (ROS) generated by spermatozoa play an important role in normal physiological processes such as sperm capacitation, acrosome reaction, oocyte fusion and stabilization of the mitochondrial capsule in the mid-piece [1, 2]. ROS produced by spermatozoa and leukocytes are scavenged by various antioxidants in the seminal plasma. Uncontrolled production of ROS that exceeds the antioxidant capacity of the seminal plasma leads to oxidative stress (OS), which is harmful to spermatozoa [1]. In addition, ROS is an independent marker of male factor infertility. ROS measurement by chemiluminescence method is highly sensitive and accurate method with low intra-observer, intra-observer variability [2-4]

Levels of ROS have been correlated with semen parameters, in all these studies a single ejaculate from each subject was examined at a single time point (2, 3). However, longitudinal studies describing the association of ROS levels with semen parameter within a fertile man are lacking. Our aim was to conduct a longitudinal study and monitor within individual variations in ROS levels and correlate this with semen parameters.

This study was approved by the Cleveland Clinic Institutional Review Board. A single normal healthy proven fertile man provided 36 semen samples over a period of 21 months (From October 2006 to June 2008) with an abstinence of 1 to 10 days.

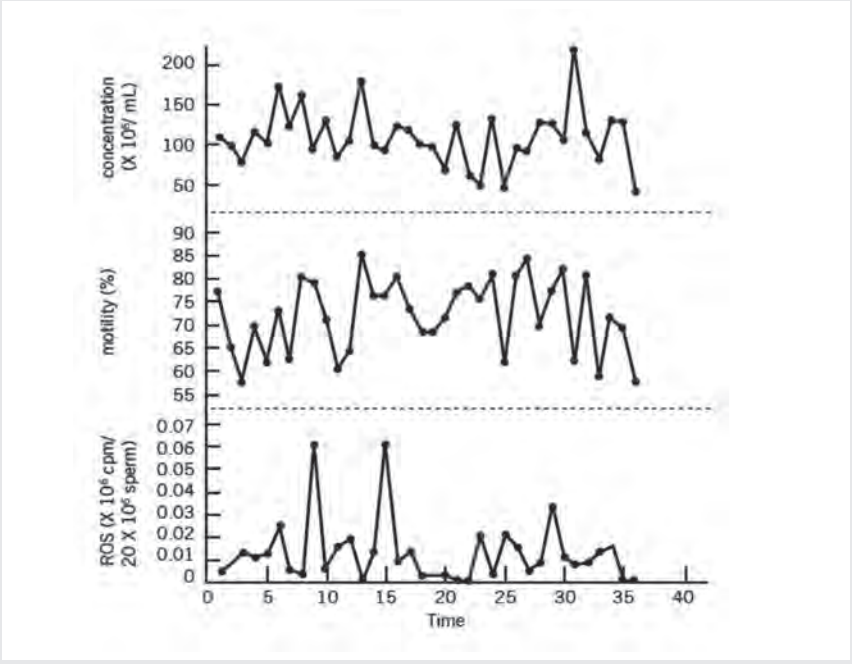
Following complete liquefaction at 37° C for 20 minutes, 5 µL of each specimen was loaded on a 20 µL Microcell chamber (Conception Technologies, San Diego, CA) and analyzed for sperm concentration and motility according to World Health Organization (WHO, 1999) guidelines [5]. Seminal smears were stained with Diff-Quick (Baxter Healthcare, McGraw Park, Ill), and sperm morphology was assessed using WHO criteria (5).

The presence of white blood cells (WBC) in the specimen was detected by the Endtz test [2]. Briefly, 20 µL volume of liquefied specimen was placed in a 0.5 mL sample cup; 20 µL of tyrodes buffer and 40 µL of benzidine solution were added. The mixture was vortexed and allowed to sit at room temperature for 5 min. Peroxidase-positive WBCs, which stain dark brown, were counted in all 100 squares of the grid in a Makler chamber under the 20X bright-field microscope. The results after correction for dilution were recorded as the number of WBCs counted x 10^6/ mL.

Seminal ejaculates that had not undergone any additional processing (neat samples) were used for ROS measurement by chemiluminescence assay using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Chemical Co., St Louis, MO). A 100 mmoL/ L stock solution of luminol was prepared in dimethyl sulfoxide (DMSO). For the analysis, 10 µL of the working solution (5 mM) was added to 400 µL of neat sperm sample. Chemiluminescence was measured for 15 min using a Berthold luminometer (Autolumat LB 953; Bad-Wildbad, Germany). Results were expressed as X 10^6 counted photons per minute (cpm)/ 20 x 10^6 sperm [4]. ROS levels >0.0185 were considered as positive based upon our lab cutoff value. Data analysis and correlation studies were done by JMP software version 7.0 for windows. P <0.05 was considered significant.

Sperm concentration (Mean ± SD) was 106.3 ± 36.3X 10^6/ mL and motility 71.6 ± 7.96 %. ROS levels ranged from 0.00 to 0.06 X 10^6 cpm/ 20 X 10^6 sperm. The mean ROS level was 0.0122 ± 0.0137 units (95% CI = 0.0169). Coefficient of variance for sperm concentration, motility and ROS were 30%, 10% and 110 % respectively. Correlation analysis demonstrates no significant correlation between ROS levels and sperm parameters. The fluctuations in sperm motility, concentration and ROS are demonstrated in figure 1. A complete semen analysis including the presence of round cells, white blood cells and sperm morphology was evaluated only during first visit and it was within normal range according to the WHO criteria.

Figure 1 Variations in concentration, motility and ROS (reactive oxygen species) in 36 semen samples provided by a single fertile donor over a period of 21 months.



Semen analysis is one of the important clinical laboratory tests to evaluate male infertility and intra individual variations in the semen parameters have been reported [6]. Reports from our center show that measurement of ROS level should be included in routine semen analysis [2, 7, 8]. Previously, we have reported that ROS cutoff value of 0.0185 X 10^6 cpm/20 X 10^6 sperm is highly predictive of infertility [2]. However, monitoring

ROS level in a fertile man and studying correlation between ROS level and sperm parameters are extremely important before oxidative stress profile can be included to evaluate infertile men.

In this study over a period of 21 months, we did not find any significant (positive or negative) correlation of ROS levels with sperm concentration and motility over a period of time within a given individual. No significant correlation of motility and concentration with ROS suggest that ROS is an independent marker of fertility in healthy fertile donor.

In the current study on a single healthy man, incidence of ROS positive seminal ejaculates was 13.8% (5 of 36 samples). In spite of positive ROS (5 samples), all (36) samples tested for Endtz test were negative **(**this means absence of any detectable peroxidase positive leukocytes in these examined specimens) [3]. In all these instances, sperm motility and concentration were not correlated with increased levels of ROS. Previously, we reported sperm motility and concentration are inversely correlated with ROS levels in infertile men. On the other hand, previous studies have linked ROS in various physiological processes of spermatozoa (i.e. sperm capacitation, acrosome reaction, oocyte fusion, and stabilization of the mitochondrial capsule in the midpiece). Therefore, we suggest that levels of ROS may rarely fluctuate within a fertile man but they do not affect the sperm concentration and motility. This may be possible due to the presence of adequate antioxidant defense mechanisms in this healthy individual. These fluctuations in ROS might also be due to physiological process. Previous reports also suggested, there might be seasonal variation in ROS level in semen [9, 10]. Based on these, we suggest that fluctuation in ROS value may be physiological or due to transient sub-clinical infection, transient abnormalities in spermatogenesis such as retention of cytoplasm or periodic presence of abnormal spermatozoa in semen.

In conclusion, we demonstrated that within a normal, healthy individual, although variations in the ROS levels are seen over an extended period, ROS levels are independent of concentration, motility and abstinence period. Our results present seminal ROS variation in a healthy individual of proven fertility that is different from the sperm concentration or sperm motility variation. This variation may be physiological, seasonal or life style related causes. However, we measured morphology and leukocyte counts in only first specimen of fertile donor. We did not measure morphology and leukocyte counts in subsequent specimens. So, further studies on many fertile donors over a long period of time are necessary to explore this phenomenon.

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Chapter 10

Discussion and general conclusions

Studies presented in this thesis were performed within outlines of a more extensive research project aimed to improve the role of reproductive laboratory in diagnosis and follow up treatment in male infertility caused by oxidative stress (OS) or apoptosis. Accurate diagnosis and follow up in male infertility will help avoiding any undesired outcome that may result from the use of damaged spermatozoon on embryos. As assisted reproductive technologies (ART) overcome the natural selection barrier, especially when intracytoplasmic sperm injection (ICSI) fertilization is used, we should be aware not to inject damaged (unhealthy) sperm which may harbor excess damage beyond the oocyte repair capacity [1].

The use of damaged spermatozoa may result in abnormal fertilization[2], low embryo quality [3], implantation failure [3], early pregnancy loss [4], or possibility of an increased risk of childhood diseases for those born after ICSI [5]. Moreover, ART quality has been intensified over the last decades by the growing concern about transgenerational genetic mutations through ART especially ICSI[5]. Therefore, the current suboptimal ART success rates may be attributed, at least in part, to the presence of apoptotic damage in sperm or in embryo post fertilization as a result of absence of natural sperm selection barriers [6].

This hypothesis has generated a motivation to develop new protocols for clinical andrology/ reproductive laboratories based on OS or apoptosis markers in male infertility. This approach represents an inevitable evolution of human fertility laboratory diagnostic services that expand to include novel molecular characteristics in addition to routine sperm parameters.

1. Routine sperm parameters, OS and apoptosis

Routine sperm parameters provide the basic diagnostic and prognostic tools for infertile men to identify those who suffer from defective spermatogenesis[7]. However, routine parameters show overlapping of sperm parameters between fertile and infertile individuals [8]. Interestingly enough world health organization (WHO, 2010) has lowered their cutoff values on sperm concentration over the last decades, but still they fail to discriminate male (sub) fertility. There is a poor correlation between routine semen analyses with fertility potential in infertile patients with border-line routine sperm parameters or in idiopathic infertility [8, 9].

There is a continuous effort to optimize the laboratory protocols aiming to predict the men's fertility potential and to diagnose accurately infertile men. Reports related oligo- astheno- and terato-zoospermia (OAT) to OS and / or abortive apoptotic induced sperm damage [10-12]. High reactive oxygen species (ROS) generation is present in ejaculates from infertile men with defective spermatogenesis, suggesting that ROS level plays a different role in conditions associated with defective spermatogenesis

such as production of immature sperm with morphologically abnormal (**chapter 3, 5 and 7**) or as in astheno-zoospermia (**chapter 4**) [13, 14]. Seminal ROS is now considered as an independent marker for male infertility (**chapter 9**) [15]. Although reports showed reference values of seminal ROS in physiological or pathological conditions, accurate interpretation of the seminal ROS lab values requires understanding of the overtime variability of seminal ROS[16, 17]. Seasonal changes may affect sperm parameters as explained by several physiological, genetic or environmental factors. Life style and environ-mental factors may affect the sperm parameters in infertile or even in healthy men[18, 19].

There is an increased demand for novel assays aiming to accurate detection of sperm pathologies and so help explaining more cases of the non-diagnosed idiopathic male infertility. Several observational and interventional studies showed that OS induced sperm damage are a major cause of defective spermatozoa function seen in infertile men. OS-induced sperm damage includes oxidation of molecular proteins, lipids and/or DNA components of the spermatozoa, oocyte or even of the developing embryos [4, 20]. These findings support our observations of the presence of high ROS levels, either one or both intra- and extra-cellular (**chapters 4 -7**), in ejaculated spermatozoa may be responsible for unhealthy male gametes with an induced damage beyond oocyte repair capacity and hence ART failure (**chapter 8**)[21].

Searching for better diagnostic tools, we reported for the first time comparison of luminometer and flow cytometry as tools for measurement of intra- cellular hydrogen peroxide and superoxide levels in basal and induced ROS conditions **(chapter 7).** In support of the abortive apoptosis theory, which was proposed as an origin of sperm DNA damage, possible biological roles of PARP in ejaculated spermatozoa has been reviewed in **chapter 2**. We have also reported that the presence of cleaved PARP, a marker of apoptosis, in ejaculated human spermatozoa was related to infertility and highly expressed in immature ones. Cleaved PARP **(chapters 2 and 3)** is an early marker of apoptotic damage, which is stimulated mainly by damaged DNA. Expression of PARP1 and its homologues was reported earlier [22], in agreement to our findings, which showed differential expression of PARP(s) with sperm damage conditions, mainly OS or chemical induced injuries. OS induced sperm damage was reported in infertile men who showed low total antioxidant capacity (TAC) in seminal plasma (**chapter 6**)[23, 24].

We reported the diagnostic criteria of seminal TAC clearly in **chapter 6** and recommended seminal TAC as a simple non-invasive clinical assay which can differentiate infertile patients from healthy or proven fertile men. There is substantial evidence from a number of interventional and observational studies that OS is a major causative factor in the increased level of DNA damage observed in spermatozoa[25, 26]. It has been found that ROS production is mainly produced by mitochondria through energy production cascades. Defective cell energy system in progenitors prevents proper chromatin

remodeling inducing abnormal maturation, morphological changes or abnormal sperm motility with DNA damage[26].

2. Clinical Implications of OS/ Apoptosis in Andrology lab

Seminal ROS levels are predictive for sperm DNA damage in infertile patients with abnormal high ROS levels produced mainly by abnormal spermatozoa and leukocytes in semen specimen[27]. In physiological conditions, seminal plasma plays a protective role for healthy spermatozoa [28]. However, in pathological conditions, seminal plasma fails to neutralize free radicals which are permeable, produced in large amount by abnormal spermatozoa (defective spermatogenesis) or by non-sperm cells (e.g. leukocytes) associated with conditions such as urogenital tract infections or malignancy[29, 30].

Seminal plasma, with low antioxidants and/ or high free radicals, in infertile men may be an additional source of damage to the healthy sperm resulted from accumulated ROS from leakage of abnormal cells that predominate in pathological conditions in semen specimens (**chapter 6**). Choice of proper tool, to detect accurately the levels of intracellular ROS, may be critical in certain patient specimens whenever false negative results should be avoided (**chapter 7**). Minimal detection limits is determined with right technology applied to proper specimen[31]. Sperm pathologies associated with OS and apoptosis may be due to genetic defects as seen in chapter 4 with idiopathic astheno-zoospermia or repeated ICSI failure report[21]. Maturation defects and environmental factors may be prominent even in infertile men without obvious genetic disorders (**chapters 8**). Variability over time of seminal ROS may reflect the net results of interactions between microenvironment and genetic in the male reproductive system (**chapter 9**). Over time changes should be a requirement for proper lab reporting of diagnostic and therapeutic services for patients in infertility centers.

Removal of seminal plasma may be enough in some pathological conditions such as presence of anti sperm antibodies, high ROS levels, or cytokines[32]. The importance of our findings is basically diagnostic for infertile couples with idiopathic infertility or with repeated ART failure. Proper diagnosis and successful prediction in ART outcome are essential to improve financial and psychological consequences on the infertile couples. Novel molecular and biologic assays should aim to offer them the possibility to understand accurately the origin of their infertility. A therapeutic potential application would be the selection of healthy spermatozoa not only based upon conventional sperm parameters but also based upon the molecular sperm markers.

Our work (**chapters 1-10**) shaded the light for better understanding of male infertility and added values for andrology lab evaluation that covers both conventional and

molecular parameters which are important for infertile couple management [9]. Our findings may be critical in sperm processing and sorting for reproduction laboratory. Through our work (**chapter 2-9**), we developed new assays and potential scores to be more able to personalize ART lab's services and to optimize diagnostic or prognostic management of infertile couples. Our work findings would also be important in improving the lab procedures to avoid any possible iatrogenic damage, such as long contact of healthy mature sperm with leukocytes or cellular debris. This thesis will optimize some lab services such as cryopreservation and processing through optimal use of antioxidants in vitro for reproductive tissues.

Seminal ROS is a novel parameter and still not standardized. Therefore, each lab should set up its own cutoff levels. Multiple studies reported various cutoff values in their patients' specimens using different diagnostic criteria or using different method with variation in sensitivity, units and limitations. However, lacking of variability of this marker over the time is critical to interpret accurately follow up of serial seminal ROS level. In **chapter 9,** we have investigated the variation of seminal ROS levels in a healthy individual over a longitudinal study for 21 months. ROS is an independent parameter from sperm concentration or sperm motility observed with time[15]. Sperm immaturity is associated with apoptosis or OS induced damage to DNA or plasma membranes. Induced sperm damage may result in fluctuation in patient's sperm motility and/ or sperm concentration. Changes in sperm quality overtime were reported to be due to seasonal or unexplained as noticed in lowering WHO reference values from 1980s to 2010[33]. Life style plays a critical role, as reported by Smits et al 2009 who reported low concentrations of folate in seminal plasma may be detrimental for sperm DNA stability[34]. Reports showed exposure to environmental conditions during work in some occupations or pollution may impact the fertility potential of the exposed men [35]. Moreover, special attention for food supplementation has been suggested for infertile couple treated with ART[36].

Interestingly enough, report shows OS induced DNA damage may be different in OS induced or apoptotic DNA damage[37]. Figure 1 shows the extensive DNA damage in apoptosis induced compared to OS induced nuclear damages after incubation with H_2O_2 (100µM) or chemical (Actinomycin D 10 ng/mL) injuries. L-carnitine supplementation at proper (0.3 mg/mL) or improper (0.6 mg/mL) high concentrations affect the extent of DNA damage in treated vs. untreated mouse embryonic cells [37]. Moreover, presence of additional OS, from improper antioxidants supplementation, to apoptosis causes more extensive nuclear damage. This may indicate that OS induced DNA damage may be limited exposed foci which will expand later following activation of apoptotic pathways inducing extensive DNA damage (Figure 1 A).OS/ apoptotic damage may affect extensively the status of spermatogenesis in men with improper antioxidant treatments. Reports showed the importance of antioxidant supplementation before and after surgery may be helpful to restore spermatogenesis status

normally[38, 39]. In-vitro study, antioxidants should be titrated (Figure 1B) and optimized to avoid iatrogenic sperm damage (Figure 1B, from un-published work).

3. Molecular assays and ART

Currently, routine sperm parameters used for ART are mainly based upon sperm concentration, motility and morphology. However, there is a possibility to use sperm containing varying degrees of damage spermatozoa especially DNA damage (**chapters 8**). In support of the impact of paternal DNA status on probability to optimize ART outcome and to achieve healthy live births, a study showed an association of sperm aneuploidies and apoptotic induced damage in infertile men with recurrent pregnancy loss[40]. It is therefore much likely that failed ART cycles and high miscarriage rates can be minimized by the use of spermatozoa with intact normal genomic status. Absence of natural selection in ART may increase the possibility of using apoptotic DNA damaged spermatozoa beyond the repair capacity of oocyte or the growing embryonic cells yielding finally to failed cycle due to low embryo quality and increased miscarriage rates [41].

This assumption is reinforced by Dada et al (2010) in describing a case report, where high ROS is associated with nuclear as well as mitochondrial DNA damage that may cause repeated failure even with normal karyotyping status (46, XY) [21]. Likewise, high ROS in embryo culture is detrimental for prediction of the embryo quality and pregnancy outcome following both ICSI/ IVF cycles in human reproduction laboratory[42].

OS-induced DNA damage may be one of the main causes of embryo fragmentation that can be avoided by the use of embryo culture media supplemented with proper dosage of antioxidants. Antioxidants protection, against OS-induced DNA damage in spermatozoa and embryos, is dose dependant and requires more studies for optimization and individualization (Figure 1) [37]. Direct and indirect evidences about the role of antioxidants supplementation along with environmental/ life style concerns are rapidly growing and may in the near future be applicable to all men requiring ART especially with progressive decline in sperm quality[43].

4. Potential application for OS/ apoptosis in diagnostic lab

a) Novel Assays/ prognostic multi-marker scores:

We support the use of seminal plasma TAC, seminal ROS assay and/ or Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for measurement of indirect or direct OS and DNA damage for diagnosis in infertility clinics. Combination of more than one assay will results in increase in specificity and/ or sensitivity for

these new prognostic scores. Such new scores may include: direct/ indirect OS score; it is formulated to cover the ratio between seminal plasma TAC levels (indirect OS, Chapter 6) with seminal ROS levels (direct OS, **chapters 5, 7-9**).

These scores will be more informative than any individual assay in the evaluation of sperm integrity as of adding values of their components. Indirect OS/ sperm DNA fragmentation score is a second approach which can be calculated using the percentage of $TUNEL^{+ve}$ sperm and seminal TAC levels. Direct OS/ sperm DNA damage score can be calculated using values of seminal ROS and % $TUNEL^{+ve}$. A comprehensive score for DNA damage consists of direct OS/ indirect OS/ sperm DNA fragmentation. The proposed multivariable score may be more specific and/ or more sensitive for evaluation of infertile men. If the prevalence of infertility is known or estimated within a particular testing population, which varies within different communities, then these multi-parameter score results could be used to estimate positive and negative predictive values (PPV and NPV) for such a population. The following equation uses the estimated sensitivity and specificity for demonstrating any prediction method of choice:

$$\textbf{Estimated PPV=} \frac{\text{(Sensitivity x Prevalence)/}}{\text{(Sensitivity x Prevalence) + [(1 - Specificity) x (1 - Prevalence)]}}$$

$$\textbf{Estimated NPV=} \frac{\text{[Specificity * (1 – prevalence)]/}}{\text{(1 - Sensitivity) x Prevalence) + [Specificity x (1 - Prevalence)]}}$$

For example if we consider prevalence of infertility is 5% in community A while it is 20% in community B. Suppose sensitivity and specificity for global semen ROS are 90% and 55% as a screening assay. Estimated PPV/ NPV will be 10/99 for community A and 33/96 for community B respectively. Similarly, in case of a specific assay such as sperm DNA damage test, if sensitivity is 60% and specificity is 95%, the estimated PPV/ NPV will be 37/98 for community A and 73/89 for community B respectively.

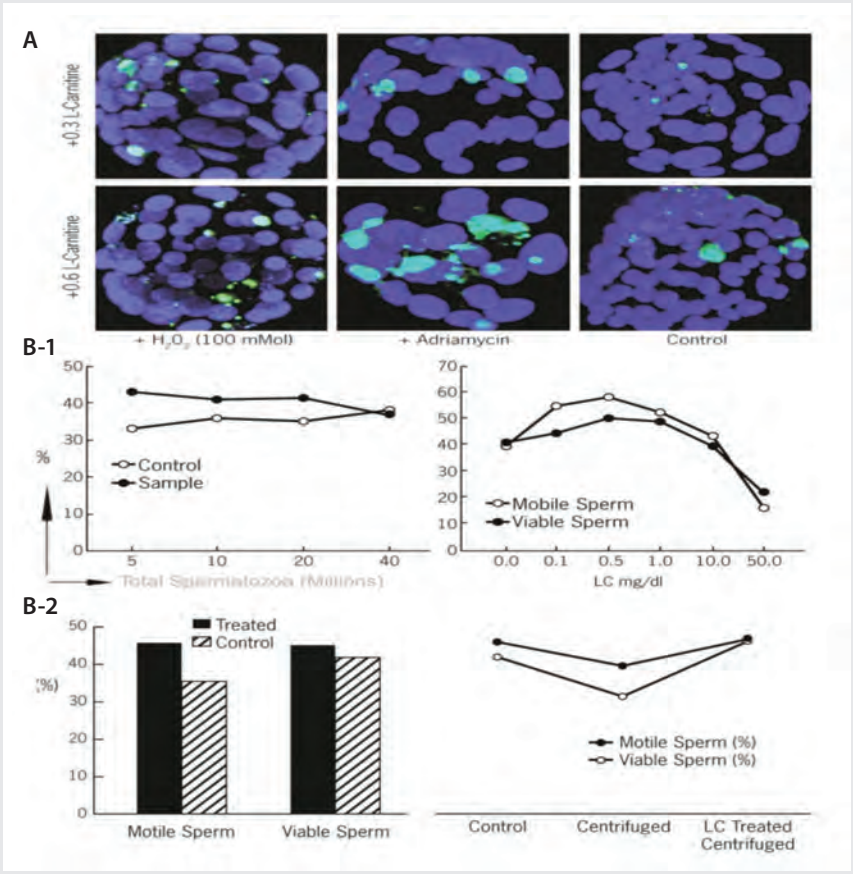
b) Optimizing ART Options/ Risk Reduction:

Proper use of our novel markers (presented in **Chapters 3-8**) or proposed multi-marker scores (mentioned above) could be helpful for management of infertile men with OS and apoptosis. Our work showed examining for OS/ apoptosis may be critical to help in both diagnosis and prognosis of infertile men for successful outcome of ART [44].

c) Therapeutic/ Diagnostic Tools:

For spermatozoa: in unpublished data, we observed that protection against OS/ apoptosis may be beneficial only if we use optimal dose of it. L-Carnitine in high concentrations may have an adverse outcome, observed through a dose-dependent decrease in sperm motility and viability (Figure 1B). Sperm motility and viability can

Figure 1 Nuclear DNA fragmentations differ in chemical induced apoptosis from OS induced damage [**A**]. Cellular response is different indicating differences in the affected pathways in both kinds of cellular injuries. The protective mechanism of antioxidant is effective only in optimal concentration at which the disturbed cellular pathways could be restored. Optimal antioxidant concentrations should be titrated according to cell concentration as well as different antioxidant concentrations [**B-1**]. Confirmation of the protective antioxidant treatment may be evaluated in-vitro to examine sperm quality improvement before processing to prevent any iatrogenic damage [**B-2**]. Data represented in this figure was from unpublished experiments.



be improved only with optimal conditions usage that may vary within individuals. Such conditions should consider the antioxidant dosage as well as the cell concentration. Cellular function may be affected by the basal ROS values and/ or extent of DNA breaks. Optimal spermatozoa function requires basal ROS levels, which is impaired when basal ROS is depleted by using high antioxidants concentrations [39].

d) Advantages and limitations of our approach

Application of our clinical pathology approach in fertility labs will improve ART outcome and pregnancy achievement of healthy live births [45]. The more accurate diagnosis the lighter burden will be upon the infertile couples. It would also improve the health services provided by infertility clinics. Therefore the use of our clinical pathology approach could result in significantly higher embryo quality and development with limited failure ART cycles [46]. Our approach is still in need to be applied and validated in different clinical settings. Different clinical diagnosis may require additional modifications for optimizing our novel markers and proposed scores for personalizing reproductive medicine in infertility clinics.

e) Future directions

Our clinical pathology approach for studies included in this thesis will serve as a basis for future projects to validate its clinical significance on large scale ART services such as IUI, IVF or ICSI. It will help in better understanding of natural achievement of pregnancy and hence to overcome recurrent pregnancy failure. Developing novel scores which cover infertile couple's clinical and lab parameters would be easily applied in the light of molecular and routine investigations discussed in this thesis.

General conclusions

Based on the results presented in this thesis, we can conclude that:

- A rise in sperm DNA damage should be expected in conjunction with immaturity of male gametes. Immature spermatozoa are rich in cytoplasmic and nuclear enzymes that may play a critical role in high ROS generation. Therefore we could easily observe OS-induced sperm damage and apoptotic like changes in ejaculated spermatozoa in patients with defects in spermatogenesis maturation.
- Novel markers (such as intracellular ROS, cleaved PARP, sperm apoptosis markers, seminal plasma TAC levels and eNOS SNP mutation) have been described in this thesis for the first time in human ejaculated spermatozoa. Theses markers may provide critical values in improving diagnostic and therapeutic managements for infertile couples serviced in reproduction lab and infertility clinics.
- Our work shaded the light upon comparisons between the diagnostic tools and/or markers to facilitate the selection and hence personalized services for the infertile couples based on their accurate diagnosis and follow up. Intracellular ROS assay more sensitive, selective and specific than global seminal ROS measurement by chemiluminescence. Intracellular hydrogen peroxide is related to apoptosis and maturity of spermatozoa. Seminal ROS is an independent parameter that may fluctuate even in healthy based upon the maturation or disease stages.

- OS induced sperm damage may be involved in multi pathologies affecting ejaculated human spermatozoa; however, it may be solely responsible in inducing sperm DNA fragmentations. So at least 10% of sperm DNA damage may be preventable through following our work's guidelines.
- Interpretation, potential application and recommendations based upon this thesis have been discussed and explained for andrology lab guidance as well as for future studies perspectives aiming to optimize infertility clinic services.

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Summary

There are many potential causes of male gametes' damage, including abortive apoptosis, oxidative stress (OS) associated with infections, environmental exposure or life style conditions, as well as spermatogenesis defects which associated with retention of excess residual cytoplasm and hence defective spermatozoa function. Reports linked presence of apoptotic characteristics and OS induced damage in human spermatozoa, resulting in fertilization failure of both in-vivo (natural or intrauterine insemination, IUI) and in-vitro fertilization (IVF). In addition OS induced damage may compromise the quality and development of early embryogenesis and hence affect the outcome of assisted reproductive technologies (ART). The importance of an accurate diagnosis of male gamete damage / early embryo quality has been intensified by the growing concern in the transmission of genetic diseases to the developing embryos through ART, especially with intra-cytoplasmic sperm injection (ICSI). The current suboptimal ART success rates may be attributed, at least in part, to the use of damaged sperm for oocyte insemination, inducing embryo damage as a result of absence of natural sperm selection barriers.

This hypothesis has motivated us to develop new diagnostic protocols to achieve an accurate diagnosis for male infertility based on OS or apoptosis-like markers in spermatozoa. Such approach represents the inevitable evolution of the reproductive laboratory diagnostics towards molecular parameters in addition to routine examination of cellular characteristics in spermatozoa.

ART provides solutions to infertile couples', especially in cases of male factor and unexplained infertility conditions. Nowadays, infertility is considered as a disease rather than an isolated condition of reproductive failure. Infertility affects 10-15 % of couples who are seeking for parenthood where male and female partners contribute equally (50%) in infertility causes. Managing infertile couples is expensive and has an unusual psychological burden. There is an increasing demand for new assays and parameters to monitor evaluate and improve ART outcome. Routine investigations may not be adequate to serve purposes of understanding the origin of male and female infertility. In addition, stress that merge with conditions such as modernization, life style and environmental changes, gene mutations and chromosomal abnormalities can disturb the highly refined biochemical events associated with spermatogenesis and/ or embryogenesis. This can ultimately lead to an abnormal chromatin structure which is incompatible with fertility [1]. Sperm nuclear chromatin abnormalities/ DNA damage could occur at the time of, or be the result of, faulty DNA remodeling at spermiogenesis [2]. Or, alternatively, it could be the result of free radical induced damage [3] or a consequence of apoptosis [4]. Sperm processing techniques are essential in ART. Spermatozoa may get iatrogenic damage during preparation. Such damage is mainly detected through new assays for apoptosis and OS.

Our thesis work focused on introducing new markers for sperm evaluation in addition to the evaluation of other parameters which may be valuable for clinical application in the reproduction laboratory. Our work results may help to improve the performance of reproduction laboratory and the IVF/ ICSI outcome in general. The studies in this thesis are grouped together into 3 parts: 1) Sperm maturity, apoptosis and OS induced damage conditions; 2) Novel markers for OS and apoptosis; 3) applications of apoptotic markers in infertility. Finally, the clinical application of our work are described and discussed with different possible limitations and precautions regarding accurate diagnosis and patient selection.

In **Chapter 1**, includes an overview of spermatogenesis with special empahsis on the origin of both apoptosis and OS in male germ cell pathologies. Conventional and possible future diagnostic approaches to improve reproduction lab services are presented. An overview of the clinical aspects of male infertility is given, which can serve as a diagnostic and therapeutic tool for infertile couples.

The second part of this thesis comprises the exploration of novel OS/ apoptotic markers expressed in male gametes in relation to external or internal sperm features such as sperm motility, viability, maturation status and sperm chromatin which constitute the main selection parameters for spermatozoa.

In **Chapter 2**: sperm immaturity may compromise the individual's fertility potential and even embryo quality after fertilization. Immature spermatozoa may be affected by oxidative stress conditions and/ or apoptotic changes during spermatogenesis and hence negatively disturbing the quality of male gametes. Poly ADP-ribose polymerase protein (PARP), has been recently found to be present in ejaculated spermatozoa. This protein plays an important role in DNA damage repair through maturation stages of human spermatozoa and in different pathological conditions. We have explored possible PARP roles that may be target for future therapeutic and diagnostic application in ART.

In **Chapter 3**: we studied levels of cleaved poly (ADP-ribose) polymerase (cPARP) in ejaculated human spermatozoa after induction of OS- or chemical- induced damage. Response of sperm to the injurious agents may be controlled by the extent of DNA damage and/ or ATP levels which reflect mainly mitochondrial function. Semen specimens were collected from eight healthy sperm donors, prepared with double-density-gradient centrifugation, and divided into aliquots: untreated (control), hydrogen peroxide (H_2O_2), H_2O_2 & 3-aminobenzamide (3-ABA), staurosporine (STS), and STS & 3-ABA respectively. Cleaved PARP was detected in both neat and mature fractions. The cPARP levels were similar in both mature and immature spermatozoa. In combined mature and immature fractions, a higher percentage of late apoptotic sperm was seen in STS + 3-ABA vs STS. Higher levels of late apoptotic spermatozoa were seen in immature versus mature fractions within STS and STS + 3-ABA groups. Lower levels of cPARP were seen in immature versus mature fractions in H_2O_2 and H_2O_2

+ 3-ABA treated groups. Cleaved PARP was related to levels of activated caspase-3. Cleaved PARP was detected in both neat and mature fractions. This suggests that cleaved PARP is present in ejaculated human spermatozoa. Poly (ADP-ribose) polymerase inhibitors may play a different role in chemical vs. OS-induced sperm damage.

In **Chapter 4** we report a single nucleotide polymorphism (SNP) of endothelial nitric oxide synthase (eNOS) gene (Glu298asp variant) in infertile men with astheno-zoospermia. We investigated the frequency of G894T polymorphism (Glu298Asp variant) within exon 7 of the eNOS gene in 70 infertile men and in 60 healthy men. Sperm kinetics were assessed with computer assisted semen analysis (CASA). A single nucleotide polymorphism (SNP) of the eNOS gene in exon 7 was determined by allele-specific polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP). Sequencing analysis was used to confirm the specific genotype. The G894T eNOS (T) allele was found at a higher frequency in patients with asthenozoospermia (60%) compared to control group (22.5%). In those men, the percentage of rapid motile sperm (grade a+b) was low in asthenozoospermic infertile men harbor homozygote's eNOS (TT) genotyping vs. those with wild-type eNOS (GG) or heterozygote's eNOS (GT) genotyping ($p = 0.01$). In the fertile men, the percentage of rapid motile sperm (grade a+b) was higher in the wild-type eNOS than in the eNOS (TT) ($p=0.03$) or eNOS (GT) genotyping ($p=0.04$). Our findings suggest that the T allele encoding for aspartic acid of the eNOS (Glu298Asp) gene may contribute to low sperm motility. Whenever defective enzymes involved in pathways generating free radicals such as H_2O_2, $O^{-\bullet}$ or NO, abnormal levels of these free radicals will compromise spermatozoa functions. Unexplained asthenozoospermia may get benefited from genetic screening for genes of certain enzymes involved in OS.

In **Chapter 5**: we have examined routine and molecular markers of OS/ apoptosis in ejaculated spermatozoa. We studied sperm viability, apoptosis, and intracellular reactive oxygen species (ROS) levels in human spermatozoa within immature and mature sperm fractions. Basically mature spermatozoa showed significantly lower incidence of apoptotic sperm and intracellular $O_2^{-\bullet}$ levels but higher amounts of intracellular H_2O_2 compared with neat semen. Therefore we were able to conclude that there is a differential shift of both intracellular H_2O_2 and $O_2^{-\bullet}$ in each sperm fractions that may affect sperm quality. Sperm apoptosis is related to intracellular H_2O_2 levels, which in turn are affected by intracellular $O^{-\bullet}$ levels.

In section 3 we focused on applying some of our novel markers for OS/ apoptosis in andrology and ART. We have applied our bench work in-vitro studies to clinical applications for patient benefits. The clinical value of the seminal plasma total antioxidant (TAC) assay for clinical lab use requires examining it in infertile versus healthy sperm donors was investigated in **Chapter 6.** ROC curve analysis was used to define new cutoff, sensitivity, specificity and ranges of the seminal plasma TAC in our

patients and control populations. Proven fertile donors showed higher TAC values (median and range): 1700 (1440 - 2290 µM); compared to the infertile patients: 1310 (1040 - 1600 µM). The cutoff to distinguish between fertile controls and infertile men was 1420 µM. At this threshold, specificity was 64% and sensitivity 76%. These observations suggest that seminal plasma TAC as measured by the colorimetric assay is a reliable and simple test for the diagnosis and management of infertile men.

In **Chapter 7**: we evaluated chemiluminescence for specific/ selective estimation of the intracellular ROS, the most common tool for seminal ROS measurement with flow cytometry. Semen samples from 18 healthy male volunteers were subjected to sperm preparation and measurement of ROS by chemiluminescence using luminol and lucigenin before and after H_2O_2 exposure and by flow cytometry using dihydroethidium (DHE) for $O_2^{\cdot-}$ and dichlorofluorescin diacetate (DCFH-DA) for H_2O_2. Seminal ROS values detected by luminol and lucigenin showed significant relationships to intracellular ROS as detected by $\%DCF^{+ve}$ and $\%HE^{+ve}$ spermatozoa. Intracellular ROS levels were detectable by flow cytometry in chemiluminescent negative samples. Both mature and immature spermatozoa had significantly high percentages of cells positive for H_2O_2. On the other hand, the percentage of $O_2^{\cdot-}$ +ve cells in un-processed semen was significantly higher compared to mature but significantly lower than in the immature sperm fractions. Therefore, we recommend ROS measurement by flow cytometry on the basis that it requires a lower sperm count, is comparable to chemilumenscence, and has higher specificity for intracellular ROS in viable spermatozoa.

In **Chapter 8:** we have explored the clinical and laboratory findings in infertile men with high or low seminal ROS conditions. We examined sperm motility, seminal plasma TAC, DNA fragmentation and medical history in 101 infertile men. Group I (n = 57) included men with seminal ROS (<250 RLU/sec/X 10^6 sperm) while group II (n = 44) included men with seminal ROS levels (≥250 RLU/sec/X 10^6 sperm). Group II had high incidence of sperm DNA fragmentation than group I. The odds ratio of 1.25 for elevated ROS levels corresponded to >10% greater DNA fragmentation in our patients (95% CI 1.01 - 1.53). Group II showed poor motility, a higher incidence of leukocytospermia and higher ROS-TAC scores compared with group I. ROS was negatively correlated with sperm curvilinear velocity (VCL) (r = -0.24), linearity (r = -0.24) and sperm motility (r = -0.31). Sperm motility was negatively correlated with $\%TUNEL^{+ve}$ sperm (r = -0.39). Therefore we concluded that a rise in seminal ROS levels by 25% was associated with a 10% increase in sperm DNA fragmentation. Sperm motility was affected by seminal ROS and sperm DNA fragmentation.

Follow up of a healthy sperm donor is reported in **Chapter 9;** in a longitudinal study over a period of 21 months, we demonstrated that seminal ROS levels are independent from sperm concentration, motility or abstinence duration within a healthy sperm donor, although some variations were observed in ROS levels. Our hypothesis is that fluctuation in seminal ROS values may be related to physiological or transient changes

in spermatogenesis. Therefore, ROS level is a unique additional parameter to semen analysis which is not affected by the abstinence period.

The main **conclusions** from this work are: 1) OS/apoptotic measures may compromise the male gametes. OS/apoptotic measurements may vary with stages of sperm development and/ or disease conditions within infertile patients for certain clinical diagnosis. 2) Novel markers and assays may be required for OS/ apoptotic measurements that could be applicable to offer additional accurate services for andrology laboratory and infertility clinic. 3) Flow cytometry is a powerful clinical pathology tool for andrology laboratory through providing a series of diagnostic services for clinical and research activities. 4) Although routine examination may be critical for infertile men, examination for DNA damage may be mandatory to explain sperm pathologies, or repeated ART failures. 5) OS may be responsible for at least 10% of sperm DNA damage in infertile men. OS may be also related with different pathologies such infection, genetic disorders or idiopathic infertility.

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Samenvatting

Er zijn talrijke mogelijke oorzaken voor de beschadiging van mannelijke gameten, zoals 'abortive' apoptose, oxidatieve stress (OS) door infecties, milieufactoren of de lifestyle-situatie dan wel fouten in de spermatogenese die gepaard gaan met het vasthouden van het overtollige resterende cytoplasma en derhalve een slecht functioneren van de spermatozoa. In rapporten werd een verband gelegd tussen de aanwezigheid van apoptotische kenmerken en door OS gerichte schade aan humane spermatozoa, met als gevolgd een mislukte bevruchting van zowel in-vivo (natuurlijke of intra-uteriene inseminaties/IUI) als ook in-vitro-fertilisatie (IVF). Bovendien kan een door OS veroorzaakte beschadiging de kwaliteit en de ontwikkeling van de embryo's compromitteren en kan derhalve invloed hebben op het resultaat van geassisteerde voortplantingstechnieken (Assisted Reproductive Technology, ART). Het belang van de juiste diagnose van de schade van het mannelijke gameet en de kwaliteit van vroege embryo's wordt nog verder benadrukt door de toenemende ongerustheid over de mogelijke overdracht van genetische aandoeningen door ART, vooral door intracytoplasmatische sperma injectie (ICSI). Het tegenwoordige suboptimale succespercentage van ART kan minstens ten dele worden toegeschreven aan het gebruik van beschadigd zaad bij de inseminatie van oöcyten, met als gevolg abnormale embryo's door het ontbreken van natuurlijke zaadcel selectiebarrières.

Deze veronderstelling heeft ons gemotiveerd om nieuwe diagnoseprotocollen te ontwikkelen om een juiste diagnose van mannelijke infertiliteit te stellen, gebaseerd op OS of apoptose markers in spermatozoa. Een dergelijke aanpak betekent de onvermijdelijke ontwikkeling van de diagnostiek in laboratoria naar zowel moleculaire parameters als ook routinematig onderzoek van andere cellulair kenmerken in spermatozoa.

ART biedt oplossingen voor paren met fertiliteitproblematiek, vooral in het geval van mannelijke subfertiliteit en onverklaarde oorzaken voor de subfertiliteit. Tegenwoordig wordt subfertiliteit als een "ziekte" beschouwd, niet als een op zichzelf staand mislukken van de voortplanting. Subfertiliteit komt voor bij 10-15% van alle paren met kinderwens. De oorzaken ervan zijn evenredig (met telkens 50%) verdeeld over mannen en vrouwen. Het begeleiden van subfertiele paren brengt hoge kosten met zich mee en betekent een bovengemiddelde psychologische belasting. Er bestaat steeds meer behoefte aan nieuwe testen en nieuwe referentiekaders voor het in kaart brengen, evalueren en verbeteren van de resultaten van ART. Routineonderzoek is vaak niet meer afdoende om de oorzaken van mannelijke en vrouwelijke subfertiliteit te begrijpen. Bovendien kan stress in combinatie met factoren zoals de modernisering, leefstijl, veranderingen van het milieu, genmutaties en chromosomenafwijkingen de zeer gevoelige biochemische processen verstoren die gekoppeld zijn aan spermatogenese en/of embryogenese. Dit kan uiteindelijk leiden tot een afwijkende

chromatinestructuur die onverenigbaar is met vruchtbaarheid.[1] Kernchromatine afwijkingen c.q. beschadigingen van het DNA van het zaad zouden zich kunnen voordoen ten tijde van of zouden het resultaat kunnen zijn van een foutieve opbouw van het DNA tijdens de spermiogenese[2]. Ze zouden ook het resultaat kunnen zijn van door vrije radicalen veroorzaakte beschadigingen[3] of een gevolg van apoptose.[4] Zaad opwerkingstechnieken zijn cruciaal bij ART. De spermatozoa kunnen tijdens de bewerking iatrogene beschadigingen oplopen. Deze beschadigingen worden voornamelijk gedetecteerd door middel van nieuwe testen voor apoptose en OS.

Het werk van ons proefschrift is gericht op het introduceren van nieuwe markers voor de evaluatie van het zaad, naast andere parameters die waardevol zouden kunnen zijn voor de klinische toepassing in voortplantingslaboratoria. Onze resultaten kunnen er mogelijk aan bijdragen door resultaten van de geassisteerde voortplanting en de resultaten van IVF/ICSI in het algemeen te verbeteren. In dit proefschrift, de uitkomsten van onze onderzoek zijn onderverdeeld in drie delen: 1) rijpheid van het zaad, apoptose en door OS veroorzaakte schade; 2) nieuwe markers voor OS en apoptose; 3) toepassingen van apoptotische markers in infertiliteit diagnosis. Tenslotte wordt de klinische toepassing van ons werk beschreven en bediscussieerd in het licht van de verschillende mogelijke beperkingen en voorzorgmaatregelen voor wat betreft de juiste diagnose en patiëntselectie.

Hoofdstuk 1 bevat een overzicht van de spermatogenese met speciale nadruk op de oorsprong van zowel apoptose als OS bij mannelijke kiemcelpathologie. Conventionele en mogelijke toekomstige diagnostische methodes om de dienstverlening van laboratoria voor geassisteerde voortplanting te verbeteren worden gepresenteerd. Er wordt een overzicht gegeven van de klinische aspecten van mannelijke infertiliteit dat kan dienen als diagnostisch en therapeutisch instrument voor onvruchtbare paren.

Deel 2 van dit proefschrift bestaat uit onderzoek naar nieuwe OS- en apoptotische markers die zich uiten in mannelijke gameten met betrekking tot uitwendige of inwendige zaadkenmerken, zoals de motiliteit van het zaad, de vitaliteit, de rijpingsgraad en het chromatine, die de voornaamste selectieparameters voor spermatozoa vormen.

In **Hoofdstuk 2** rapporteren wij dat de onrijpheid van het zaad het vruchtbaarheidspotentieel van het individu en zelfs de kwaliteit van de embryo na de bevruchting kan compromitteren. Onrijpe spermatozoa kunnen door oxidatieve stress en/of apoptotische veranderingen gedurende de spermatogenese worden aangetast, en kunnen derhalve de kwaliteit van de mannelijke gameten verstoren. Recent werden poly-ADP-ribose-polymerase-proteïnes (PARP) in geëjaculeerde spermatozoa gevonden. Dit proteïne speelt een belangrijke rol bij het herstellen van beschadigingen van het DNA door de rijpingsfases van humane spermatozoa en onder verschillende pathologische condities. We hebben de mogelijke rol van PARP onderzocht die een doel-

stelling zou kunnen zijn voor toekomstige therapeutische en diagnostische toepassingen in ART.

In **Hoofdstuk 3** hebben we de niveaus van "cleaved"poly-(ADP-ribose)-polymerase (cPARP) in geëjaculeerde humane spermatozoa na de inductie van OS of chemisch geïnduceerde beschadigingen onderzocht. De respons van het zaad op de agenten die beschadigingen veroorzaken zou gecontroleerd kunnen worden door de omvang van de DNA-schade en/of de niveaus van het ATP, die voornamelijk het mitochondriale functie weerspiegelen. Zaadmonsters van acht gezonde zaaddonoren werden verzameld, opgewerkt met gradient centrifugeren en verdeeld in aliquoten: respectievelijk onbehandeld (controle), waterstofperoxide (H_2O_2), H_2O_2 & 3-aminobenzamide (3-ABA), staurosporine (STS) en STS & 3-ABA. Cleaved-PARP werd zowel in de oorspronkelijke als in de opgewerkte fracties gedetecteerd. De cPARP-niveaus in de rijpe en de onrijpe spermatozoa waren soortgelijk. In gecombineerde rijpe en onrijpe fracties werd een hoger percentage apoptotisch zaad in STS + 3-ABA versus STS gezien. Hogere niveaus van 'late apoptotische spermatozoa werden gezien in onrijpe tegenover rijpe fracties binnen de STS and STS + 3-ABA -groepen. Lagere niveaus van cPARP werden gezien in onrijpe tegenover rijpe fracties in met H_2O_2 en H_2O_2 + 3-ABA behandelde groepen. Cleaved-PARP is gerelateerd aan niveaus van geactiveerde caspase-3. Cleaved-PARP werd zowel in oorspronkelijke als in rijpe fracties gedetecteerd. Dit suggereert dat c-PARP in geëjaculeerde humane spermatozoa aanwezig is. Poly-(ADP-ribose)-polymerase-remmers zouden mogelijk een andere rol kunnen spelen bij chemisch geïnduceerde tegenover OS-geïnduceerde schade van het zaad.

In **Hoofdstuk 4** wordt de aanwezigheid van een enkel nucleotide polymorfisme (SNP) van een endotheel-nitraatstikstofoxide-synthase (eNOS)-gen (Glu298asp variant) in onvruchtbare mannen met astenozoöspermie gemeld. Wij onderzochten de frequentie van G894T-polymorfisme (Glu298Asp variant) binnen exon 7 van het eNOS-gen bij 70 onvruchtbare mannen en bij 60 gezonde mannen. De zaadkinetiek werd beoordeeld door middel van een door de computer ondersteunde zaadanalyse (CASA). Een singel nucleotide polymorfisme (SNP) van het eNOS-gen in exon 7 werd nader bepaald door een allele-specifieke polymerase-chain reaction (PCR-RFLP), gevolgd door 'restriction fragment length polymorphism'. Een sequentieanalyse werd gebruikt om het specifieke genotype te bevestigen. De G894T eNOS (T)-allel werd vaker gevonden bij patiënten met astenozoöspermie (60%), vergeleken met de controlegroep (22,5%). Bij deze asthenospermie mannen, een lage percentage snel progressive zaad (graad a+b) werd gevonden, zijn homozygote eNOS (TT)-genotypering in vergelijking met controle mannen met het wild-type eNOS (GG) of heterozygote eNOS (GT)-genotyping ($p = 0.01$). In de vruchtbare mannen,het percentage snel motiel zaad (graad a+b) hoger bij het wild-type eNOS dan bij de eNOS (TT) ($p=0.03$) of de eNOS (GT) genotypering ($p=0.04$). Onze bevindingen suggereren dat de T-allele-codering voor aspartiaan zuur van het eNOS (Glu298Asp)-gen tot een lage

zaadmotiliteit zou kunnen bijdragen. Wanneer er gebrekkige enzymen betrokken zijn bij trajecten die vrije radicalen als H_2O_2, $O^{-\cdot}$ of NO genereren, dan zullen abnormale niveaus van deze vrije radicalen de spermatozoafuncties schaden. Onverklaarde astenozoöspermie zou profijt kunnen hebben van genetische screening van de genen van bepaalde enzymen die bij OS betrokken zijn.

In **Hoofdstuk 5** hebben we de routine en de moleculaire kenmerken/markers van OS en apoptose in geëjaculeerd zaad onderzocht. We hebben de vitaliteit van het zaad, de apoptose en de intracellulaire niveaus van de reactive oxygen species(ROS) in humane spermatozoa in onrijpe en in rijpe zaadfracties onderzocht. Zaadmonsters van twaalf gezonde donoren werden verzameld en opgewerkt met dichtheidsgradient centrifugatie. Rijpe spermatozoa toonden een significant lagere incidentie van apoptotisch zaad en intracellulaire O_2-niveaus, maar grotere hoeveelheden intracellulaire H_20_2, vergeleken met het oorspronkelijke zaad. Wij hebben wij kunnen concluderen dat er in iedere zaadfractie een differentiële verschuiving van zowel intracellulaire H_2O_2 als ook $O_2^{-\cdot}$ plaatsvindt die de kwaliteit van het zaad kan beïnvloeden. Zaad apoptose is gerelateerd aan de intracellulaire H_2O_2 - niveaus, die op hun beurt door de intracellulaire $O_2^{-\cdot}$ - niveaus worden beïnvloed.

In sectie 3 hebben we ons gericht op het toepassen van onze nieuwe markers voor OS/ apoptose op de andrologie lab en ART. We hebben ons in-vitro-onderzoek klinisch toegepast in het belang van de patiënt. De klinische validatie van de semen plasma-totaaloxidantanalyse (TAC) assay voor het klinische laboratorium is noodzakelijk in het fertiliteitsonderzoek. Dit is onderzocht in **Hoofdstuk 6**. Er werd gebruik gemaakt van een ROC-curve analyse om de nieuwe cut-off waardes, de sensibiliteit, de specificiteit en ranges van het seminal-plasma-TAC bij onze patiënten en bij controlepopulaties te definiëren. Bewezen vruchtbare donoren toonden hogere TAC-waarden (mediaan en range): 1700 (1440 - 2290 µM); vergeleken met de onvruchtbare patiënten: 1310 (1040 - 1600 µM). De cutoff waarde voor vruchtbare controlepersonen (of onvruchtbare mannen) was 1420 µM, met een specificiteit 64% en een van sensibiliteit 76%. Deze bevindingen suggereren dat het seminale-plasma-TAC, zoals het wordt bepaald bij colorimetrische analyse, een betrouwbare en simpele test is voor de diagnose van onvruchtbare mannen.

In **Hoofdstuk 7** hebben we de chemiluminescentie voor de specifieke/selectieve schatting van het intracellulaire ROS geëvalueerd, voor de meting van seminale ROS met flowcytometrie. Zaadmonsters van gezonde mannelijke vrijwilligers werden onderworpen aan zaadbewerking en meting van het ROS door middel van chemiluminescentie, waarbij luminol and lucigenin werden gebruikt vóór en na de blootstelling aan H_2O_2 en met flowcytometrie voor dihydroethidium (DHE) voor $O_2^{-\cdot}$ en dichlorofluorescinediacetaat (DCFH-DA) voor H_2O_2. De seminale ROS-waarden toonden significante relaties met intracellulaire ROS (%DCF-positieve and %HE-positieve spermatozoa). De intracellulaire ROS werden gemeten met flowcytometrie

in chemiluminescentienegatieve zaadmonsters. Zowel de rijpe als ook de onrijpe spermatozoa hadden significant hogere percentages van H_2O_2-positieve cellen. Anderzijds was het percentage $O_2^{\cdot-}$ - positieve cellen in onbewerkt zaad significant hoger in vergelijking met de rijpe zaadfracties, maar significant lager dan dat in de onrijpe zaadfracties. Derhalve adviseren wij een ROS-meting door middel van flow-cytometrie aan, gebaseerd op het feit dat men daarvoor een kleinere hoeveelheid zaad nodig heeft, dat deze vergelijkbaar is met chemiluminescentie, en dat deze een hogere specificiteit voor intracellulair ROS in levensvatbare spermatozoa heeft.

In **Hoofdstuk 8** hebben we de klinische bevindingen en de laboratoriumuitslagen van onvruchtbare mannen met hoge of lage seminale ROS onderzocht. We hebben de motiliteit van het zaad, het seminale-plasma-TAC, de DNA-fragmentatie en de medische voorgeschiedenis van 101 onvruchtbare mannen onderzocht. In groep I (n = 57) waren mannen opgenomen met seminale ROS waarden <250 RLU/sec/X 10^6 zaad, in groep II (n = 44) mannen met een seminale ROS-waarde ≥250 RLU/sec/X 10^6 zaad. Groep II had een hogere incidentie van DNA-fragmentatie van het zaad dan groep I. Verhoogd ROS-waarde had een odds ratie van 1.25 (95% CI 1.01 - 1.53) in patiënten met >10% hogere DNA-fragmentatie. Groep II toonde een slechte motiliteit, een hogere incidentie van leukocytospermie en hogere ROS-TAC-scores vergeleken met groep I. ROS was negatief gecorreleerd aan de curvilineaire snelheid van het zaad (VCL) (r = -0.24), de lineariteit (r = -0.24) en de motiliteit van het zaad (r = -0.31). De motiliteit van het zaad was negatief gecorreleerd aan %TUNEL- positief zaad (r = -0.39). We kunnen concluderen dat een toename met 25% van het seminale ROS gekoppeld is aan een toename met 10% van de DNA-fragmentatie van het zaad. De motiliteit van het zaad werd beïnvloed door het seminale ROS en door de DNA-fragmentatie van het zaad.

De follow-up van een gezonde zaaddonor wordt in **Hoofdstuk 9** beschreven. In een lange-termijn-onderzoek over een periode van 21 maanden hebben wij aangetoond dat het seminale ROS-waarde onafhankelijk is van de zaadconcentratie, de motiliteit en de duur van de onthouding, alhoewel er wel wat variaties van de ROS-waarden werden waargenomen. Onze hpothese is dat een schommeling van de seminale ROS-waarden mogelijk gerelateerd zou kunnen zijn aan fysiologische veranderingen of aan veranderingen in de spermatogenese. De ROS-waarde zelfs is bij gezonde individuen een unieke additionele parameter voor de zaadanalyse die niet wordt beïnvloed door de onthoudingsperiode.

De belangrijkste **conclusies** van onze studie zijn: 1) OS/apoptose kunnen de mannelijke gameten compromitteren. Metingen van de OS/apoptose kunnen variëren door verschillende stadia van de zaadontwikkeling en/of omstandigheden van ziekte bij onvruchtbare patiënten. 2) Nieuwe markers voor metingen van de OS/ apoptose kunnen een waardevolle toevoeging zijn in de diagnose van mannelijke onvruchtbaarheid en kunnen additionele informatie voor behandeling bieden. 3) Flowcytome-

trie is een krachtig instrument voor andrologische laboratoria voor klinische diagnostische testen diensten en onderzoeksactiviteiten. 4) Routinematig onderzoek kan cruciaal zijn voor sommige onvruchtbare mannen, DNA-schade onderzoek is van belang bij herhaaldelijk mislukken van ART. 5) OS kan verantwoordelijk zijn voor tenminste 10% van de DNA-schade van het zaad bij onvruchtbare mannen. OS kan ook worden gerelateerd aan andere aandoeningen, zoals infecties, genetische aandoeningen of idiopathische onvruchtbaarheid.

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I have had the valuable opportunity to learn and be trained at Department of Clinical Pathology, Faculty of Medicine, Menoufyia University, Egypt. I am greatly thankful to all my Professors and Staff members for exceptional support and generous help.

Curriculum Vitae

Dr. Reda Zakria **Mahfouz** was born in El-Menoufyia, Egypt in 1972. He graduated from Shebin Elkom Faculty of Medicine, Egypt in 1996 with a very good grade and Honors. He completed his residency in Clinical Pathology, El-Menoufyia University Hospitals in 2001. He also received his Master degree from El-Menoufyia Faculty of Medicine in 2001. He completed his four year post-graduate training at Center for reproductive Medicine, Cleveland Clinic, Cleveland, Ohio where he worked as a Research Fellow. He received 5 grants from the Cleveland Clinic Research Programs Council to fund his male infertility research. He is also the recipient of the National Institutes of Health (NIH) Travel award. Dr. **Mahfouz** has published over 30 scientific papers and review articles in peer-reviewed scientific journals, authored 1 book chapter, and has presented more than 60 abstracts at both national and international scientific meetings. He is an ad hoc reviewer for several esteemed scientific journals such as the Human Reproduction, Asian J of Andrology, Andrologia, Journal of Andrology, Fertility & Sterility and European Journal of Obstetrics & Gynecology and Reproductive Biology. Dr. **Mahfouz** currently serves as a research associate in Cleveland Clinic, Ohio, USA with NIH grant to develop novel biomarkers and drug discovery.

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List of abbreviations

3AB	3- aminobenzamide
ART	Assisted reproductive technologies
BDR	blastocyst development rates
CA3	Chromomycin A3
CAD	caspase-activated deoxyribonuclease
CASA	computer assisted semen analysis
CP	Caspase
cPARP	poly (ADP-ribose) polymerase cleavage
DCF	Dichlorofluorescin
DFI	DNA fragmentation Index
DHE	dihydroethidium
DRA	DNA repair activity
eNOS	endothelial nitric oxide synthase
EPS	Externalization of phosphatidylserine
FACS	fluorescent activated sperm cell sorting
FCM	Flow cytometry
FCM	Flow Cytometry
FL	fluorescent light
FSc	Forward scatter
HDS	high DNA-stainable
ICSI	Intracytoplasmic sperm injection
IUI	Intrauterine insemination
IVF	In-vitro fertilization
MMP	mitochondrial membrane potential
NADPH	β-nicotinamide adenine dinucleotide phosphate
NF-PICS	Nuclear-Fast Red and picroindigocarmine staining
OS	Oxidative Stress
PARP	poly (ADP-ribose) polymerase
PCR-RFLP	polymerase chain reaction followed by restriction fragment length polymorphism
PMT	photomultiplier tubes
PR	pregnancy rates
PUFA	polyunsaturated fatty acids
ROC	Receiver operating Curve
ROS	reactive oxygen species
RPL	recurrent pregnancy loss
SCD	Sperm Chromatin Dispersion
SCSA	Sperm Chromatin Structure assay
SDI	sperm deformity index
SNP	single nucleotide polymorphism
SSc	Side Scatter
STS	staurosporine
TAC	total antioxidants capacity
TB	Toluidine Blue
TBA	thiobarbituric acid
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
WHO	World Health Organization

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